IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Maher M. Haddad
Art Unit	:	1644
Applicants	:	Nasser Chegini, James Burns, Michael P. Diamond, Lena E. Holmdahl
Serial No.	:	09/787,144
Filed	:	March 13, 2001
For	:	Prevention of Adhesions

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF NASSER CHEGINI, Ph.D., UNDER 37 C.F.R. §1.132

Sir:

I, Nasser Chegini, Ph.D., of the University of Florida, hereby declare:

THAT, my *curriculum vitae* is attached hereto as Exhibit A;

THAT, I am a named inventor on the above-referenced patent application;

THAT, I have read and understood the specification and claims of the subject application and the Office Actions dated January 13, 2003; July 29, 2003; and March 23, 2004;

AND, being thus duly qualified, do further declare:

1. The claims in the above-identified patent application have been rejected under 35 U.S.C. §112, first paragraph, as being non-enabled by the specification. As discussed during the telephonic interview of May 20, 2004, at issue is whether the method of the invention would function to reduce adhesion formation. I believe that a more complete understanding of the invention can be provided by an explanation of adhesion formation.

2. Adhesions are abnormal fibrous scar tissues that create connections between tissues or organs that are normally separated, or between tissues and foreign materials (such as sutures, lint, and implants). In addition to restricting movement and distorting internal organs, adhesions can

contribute to infertility, chronic pelvic pain, bowel obstruction, and difficulty in reoperative procedures. It has become clear that excess production and deposition of the extracellular matrix (ECM) which occurs during normal wound healing is a key factor in producing tissue fibrosis, including the development of adhesions. A coordinated balance between the production of the endoproteases known as matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) is an important step in tissue remodeling. In general, MMPs are not expressed constitutively *in vivo* in adult tissues, but they are induced in response to various stimuli, including pro-inflammatory cytokines, growth factors, and hormones. MMPs are also induced in tissues that normally undergo extensive remodeling, such as the endometrium during the menstrual cycle and wounds during healing. Normally, the MMPs function to degrade protein and regulate cell migration. For example, MMP-1 degrades collagens I-III and VII and fibronectin. In contrast to MMPs, the expression of TIMPs is wide spread in many tissues and is regulated in co-ordination with MMPs. TIMP-1 inhibits the activity of MMPs by forming a high affinity complex in a 1:1 ratio. In addition to inhibiting the activity of MMPs, TIMPs have also been shown to have growth factor-like activity by stimulating cell growth. Thus, for normal healing to occur, the availability of these molecules must be optimal. Inhibition, interruption, or excess expression of these molecules seems to be responsible for failure in normal healing, resulting in either impairment of tissue formation (a non-healing or chronic wound) or excess tissue formation (scar/adhesion development). Further information regarding adhesions can be obtained from the review paper submitted herewith as Exhibit B, of which I am the author (*Frontiers in Bioscience*, e91-115, April 1, 2002).

3. Several issues with regard to the enablement of the invention were discussed during the telephonic interview. The Reviewer asked under what circumstances adhesions can form, and whether TIMP-1 has been shown to be over-expressed in each case. Adhesions can be induced by infection, hemorrhage, foreign bodies, ischemia, and/or trauma (such as surgical injury). Even minimal trauma, as from the most routine activities of surgery including cutting, coagulation, and suturing can directly result in the formation of adhesions. Although intra-peritoneal or intra-

abdominal adhesions are the most common, adhesions can occur, for example, following musculoskeletal surgery, ophthalmic surgery, orthopedic surgery, surgery of the central nervous system, and cardiovascular surgery. Foreign bodies such as sutures, lint from surgical sponges, or powder from surgical gloves can cause a local inflammatory response, which can lead to an imbalance of key molecules (*e.g.*, MMPs and TIMPs) and adhesion formation. Blood present in the operating field as well as blood from other tissues can cause adhesion formation. Post-operative bleeding can cause formation of adhesions. Constriction or pinching of blood vessels may also induce the formation of adhesions.

4. It is still poorly understood why adhesions form more frequently in an individual tissue and/or patient than in others. Intra-peritoneal adhesions are more often reported because surgeons routinely conduct peritoneal dialysis, laparotomy, and laparoscopy, which frequently induce adhesion formation. Furthermore, the serosal surface of intra-peritoneal organs and the parietal peritoneum together comprise the largest surface in the body, almost equaling that of the skin. Complications of intra-peritoneal adhesions are also quite severe (*e.g.*, bowel obstruction, infertility, and chronic pelvic pain), creating a great deal of interest in prevention of intra-peritoneal adhesion development. For example, administration of anti-PAI-1 antibodies has been reported to reduce the incidence of adhesion formation in surgically-induced peritoneal injury (Falk K. *et al.*, *Br. J. Surg.*, 88:286-289 (2001), submitted herewith as Exhibit C). Peritoneal wound healing is not dissimilar from wound healing in other soft tissues of the body, however. In fact, it is well understood that a local inflammatory response and tissue remodeling are underlying processes which occur during normal wound healing throughout the soft tissues of the body, including the peritoneal cavity. It is also generally accepted that failure of these processes to properly orchestrate is necessary for formation of adhesions, whether the adhesion is induced by infection, ischemia, or trauma. The outcome is the same regardless of stimulus or anatomical site—fibrous scar tissue (adhesion). Thus, surgical adhesions of the peritoneal cavity are the accepted experimental model for adhesions in general. MMPs are typically not constitutively expressed; however, they are induced in tissues that normally

undergo extensive remodeling, such as wound tissue, and in response to various inflammatory conditions. In contrast to MMPs, the expression of TIMPs is widespread in many tissues and is normally regulated in coordination with MMP expression (see, for example, Parks, W.C., *Wound Repair Regen.* 7:423-432 (1999); Lund L.R. *et al.*, *EMBO J.*, 18:4645-4656 (1999); and Gomez D.E. *et al.*, *Eur. J. Cell. Biol.*, 74:111-122 (1997); the full-text or abstracts of which are submitted herewith as Exhibits D-F, respectively, for the Reviewer's consideration). To my knowledge, whether TIMP-1 is over-expressed in adhesions other than intra-peritoneal adhesions has not been reported in the scientific literature. However, because TIMP-1 is expressed in a wide variety of tissues, inhibiting MMP proteolytic activity and regulating cell migration, and because adhesions form as a result of excessive cellular migration and ECM deposition, there is no reason to doubt the applicability of the method of the subject invention to reduce adhesions in general, whether they are induced by infection, ischemia, or trauma, and regardless of anatomical site.

5. During the telephone interview, the Reviewer raised the question as to why the level of TIMP-1 expression in tissues and peritoneal fluid did not correlate, and why TIMP-1 expression in peritoneal fluid was not higher in patients with moderate and mild adhesions compared to patients with no adhesions. As indicated at page 7, lines 9-15, adhesions were classified on the basis of their severity. Adhesions involving only a small area (usually the fallopian tubes or ovaries) and lysed with ease were categorized as "minor". Adhesions involving larger areas were classified as "moderate". More vascular and cohesive adhesions were categorized as "extensive". The highest level of TIMP-1 expression was found in peritoneal fluid, followed by adhesions, intra-peritoneal tissues and organs (*e.g.*, large bowel, uterus, fallopian tube, ovary, peritoneum, omentum), skin, and fascia (as indicated at page 8, lines 13-15 of the patent application). With respect to the type of adhesions, the peritoneal fluid of patients with extensive adhesions had substantially higher TIMP-1 levels. Compared to peritoneal fluid, parietal peritoneum from all patients express more MMP-1, but significantly lower TIMP-1, with both expressing equal amounts of MMP1/TIMP-1 complex, as indicated at page 8, lines 23-26, of the patent application. As indicated at page 8, lines 16-17, of the

patent application, we observed that in the adhesions, the level of TIMP-1 expression was substantially higher in patients with an extensive adhesion, compared to moderate to mild adhesions, but was not significant (statistically).

6. There is substantial variability in the expression of many factors among patients and this also applies to TIMP-1. Subjecting the mean levels of TIMP-1 from these groups to statistical analysis revealed that, despite differences in their values, their mean values did not reach the desired P values because of large SEM. The difference in TIMP-1 levels in peritoneal fluid among patients with mild, moderate, and extensive adhesions, is not surprising, however. Factors detected in peritoneal fluid, such as TIMP-1, include those derived from the peritoneal cell lining (*e.g.*, mesothelial cells) and resident cells of the peritoneal fluid (*e.g.*, inflammatory and immune-related cells). Furthermore, peritoneal fluid has a periodic turnover. Thus, the level of TIMP-1 in peritoneal fluids is influenced by variables such as the expression and release of factors by the resident cells of the peritoneal fluid, the contribution of serum-derived factors to the peritoneal fluid content, the rate of peritoneal fluid turnover (and, thus, the timing of the sampling), making the data more informative as to the peritoneal environment in general, but less specific than tissue samples taken from the serosal tissue surface, where adhesions actually form. Peritoneal fluid turnover and the variability of factors present in peritoneal fluid are well documented (see, for example, Ho-dac-Pannekeet M.M., *Adv. Ren. Replace Ther.*, 5(3):205-211 (1998); Wang T. *et al.*, *Perit. Dial. Int.*, 19 Suppl. 2:S212-216 (1999); Koks C.A. *et al.*, *Fertil. Steril.*, 73(3):604-612, (2000); Hellebrekers B.W. *et al.*, *Thromb. Haemost.*, 84(5):876-881, (2000); Scheingraber S. *et al.*, *Am. J. Surg.*, 181(4):301-308 (2001); Zeier M. *et al.*, *Kidney Int.*, 63(1):298-305 (2003); the full-text or abstracts of which are submitted herewith as Exhibits G-L, respectively, for the Reviewer's consideration). Therefore, the difference in TIMP-1 levels in peritoneal fluid compared to tissue, and in peritoneal fluid among patients with mild, moderate, and extensive adhesions, is not surprising. In addition, it should be noted that the level of TIMP-1 in serosal tissues may be more relevant to intervention using a function-blocking antibody because there is more unbound TIMP-1 (*i.e.*, not complexed with MMP-1) in the tissues,

and unbound TIMP-1 is more susceptible to recognition and interference by the anti-TIMP-1 antibody administered in accordance with the method of the invention.

7. Submitted herewith for the Reviewer's consideration, as Exhibits M and N, respectively, are two publications, Chegini *et al.*, *Fertility and Sterility*, 76(6):1212-1219 (December 2001); and Chegini *et al.*, *Fertility and Sterility*, 76(6):1207-1211 (December 2001), which contain experimental data contained within the patent application and additional data. As shown in Figure 4B of Exhibit M, in an evaluation of ten patients with adhesions, the TIMP-1 protein content in adhesions was significantly higher than that in intact parietal peritoneum (P=.05) and skin (P=.03). Figure 5C of Exhibit M shows that adhesions have a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum, supporting the premise that adhesions form due to an environment that favors matrix deposition rather than degradation. Figure 2 of Exhibit N also provides evidence that the peritoneal fluid levels of TIMP-1 in subjects with extensive adhesions are higher compared with those in subjects with adhesions.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: _____
Nasser Chegini, Ph.D.

Date: 7/23/04

Exhibit A

NASSER CHEGINI, Ph.D.
CURRICULUM VITAE

Personal Information

Date of Birth	September 11, 1949
Marital Status	Married: Cherry R. Chegini
Children:	Claudine J. Chegini
Social Security Number	404-17-8915
Institution Address	Department of Obstetrics and Gynecology Division of Reproductive Endocrinology and Infertility Institute for Wound Research University of Florida College of Medicine Box 100294, JHMHC, Gainesville, Florida 32610
Business Phone Fax E. Mail	(352) 392-3929 (352) 392-6994 cheginin@obgyn.ufl.edu
Home Address	6813 NW 90th Street Gainesville, Florida 32606
Home Phone E.Mail	(352) 374-9694 nchegini@bellsouth.net

Educational Background

1973	B.Sc. (Hons), Major: Cell Biology .The National University of Iran, Tehran, Iran.
1980	Ph.D. Major: Cell & Molecular Biology, University of Southampton, Southampton, England.

Professional Positions

1973-75	Second Lieutenant, Served National Service as a Teacher in Secondary Schools in Iran
1980-81	Postdoctoral Fellow, Department of Biology, University of Southampton, Southampton, England

Professional Positions Continued

1981-87	Postdoctoral Research Associate, Reproductive Endocrinology Laboratories, Department of Obstetrics & Gynecology University of Louisville School of Medicine, Louisville, Kentucky
1987-88	Instructor Reproductive Endocrinology Laboratories Department of Obstetrics & Gynecology, University of Louisville School of Medicine, Louisville, Kentucky
1988-89	Assistant Professor Reproductive Endocrinology Laboratories, Department of Obstetrics & Gynecology, University of Louisville School of Medicine, Louisville, Kentucky
1989-1993 1989-1994	Assistant Professor Department of Obstetrics & Gynecology, Division of Reproductive Endocrinology and Infertility, University of Florida, College of Medicine, Gainesville, Florida
1993-1998	Associate Professor Department of Obstetrics & Gynecology, Division of Reproductive Endocrinology and Infertility, University of Florida, College of Medicine, Gainesville, Florida
1994-1998	Associate Professor (Adjunct) Department of Anatomy and Cell Biology, University of Florida, College of Medicine, Gainesville, Florida
1998-present	Professor Department of Obstetrics & Gynecology, Division of Reproductive Endocrinology and Infertility, University of Florida, College of Medicine, Gainesville, Florida
1998-present	Professor (Adjunct) Department of Anatomy and Cell Biology, University of Florida, College of Medicine, Gainesville, Florida

Current research activity

Our laboratories research activities focuses on two areas

1: The role inflammatory and immune related cytokines in human reproductive tract tissues in normal and disease status (leiomyoma, endometriosis and endometrial caner).

2: The role of inflammatory and immune related cytokines in peritoneal tissue repair and adhesion formation and prevention.

Research is underway to elucidate the menstrual cycle dependent expression of specific cytokines and their receptors in normal uterine tissues, and in progestin-dominant contraceptive users experiencing irregular bleeding, or women with leiomyomas and endometriosis undergo hormone therapy (i.e. GnRHa) as part of their medical management. Using in vitro cell culture models we are investigating the regulation of these genes by ovarian steroids, GnRHa, SERM and SPRM, and identify signal transduction pathways activated by their receptors and crosstalk with cytokine receptors signaling, and downstream gene expression whose products influence the outcome of these abnormalities. Using antisense oligonucleotides, viral vectors and SiRNA technology we are further investigating the role of these gene products in abnormal uterine bleeding, leiomyomas and endometriosis, and as alternative tools for their medical management.

In regard to peritoneal adhesion formation, surgically-induced adhesion models in rodents are used to identify the molecular marker for this pathological condition. Although a majority of adhesions are formed following abdominal surgery, they can be developed due to intra-abdominal infection, pelvic inflammatory diseases and endometriosis. Particular attention is being paid to growth factors such as TGF-β since it is as key cytokine directly implicated in scar tissue formation in various tissues throughout the body. Investigations are also underway to elucidate the identity of several novel genes discovered in our laboratory using differential display and microarray technologies, involving leiomyomas and adhesions. These genes or their products may serve as a marker for early detection of these tumors and identify individuals how are prone to form scars.

Grants (Active)

Chegini, N. (PI) 2001-2005	Molecular mechanism of leiomyoma growth and regression. NIH RO1HD37432. Direct cost $630,000
Chegini, N. (PI, University of Florida) Archer, D. (PI, Eastern Virginia Medical School) 2002-2007	Irregular Uterine Bleeding in Progestin-only contraceptors. NIH, RO1HD43175. Direct cost $1,250,000.
Shiverick KT (PI) et al; Chegini, N. Co-PI 2000-2005	Placental, Uterine and Prostatate effects of Organochlorines. NIH, RO1 ESO 7375. Direct cost $875,000.
Schultz GS (PI) et al. Chegini N. (co-invest). 2002-2007	Regulation of stromal wound healing by growth factors. NIH RO1NEI Direct Cost $1.125,000.00

Grants (Pending)

Chegini N (PI) 2004-2009	Uterine Molecular environment and irregular bleeding NIH RO1HD45432

Grants (Funded)

Chegini, N. (PI) 1997-2001	Cytokines/MMPs mediated peritoneal adhesion formation. The role of latex proteins. Regent Medical, Ltd. Direct Cost $260,000.00
Chegini, N. (PI) 1997-2000	Identification of molecular marker for peritoneal adhesion formation. Genzyme Inc. Tissue Repair Division. Direct cost $275,000.00.
Schultz GS (PI) et al. Chegini N. (co-invest). 1997-2001	Regulation of stromal wound healing by growth factors. NIH. RO1 NEI. Direct Cost $742,759.00
Chegini N. 1995-1996	Matrix metalloproteinases in peritoneal adhesion formation. Regent Medical, Ltd. Direct Cost $45,000.00
Chegini N. (PI) 1993-1994	The effect of surgical glove powders on peritoneal adhesion formation and cytokine production. Regent Medical, Ltd. Direct Cost $61,220.00
Yu LT (PI) and Chegini N (Co-PI) 1993-1994	Study of in vitro Bioglass™ - growth factor binding. US Biomaterials. Direct Cost $9,550.
Shiverick, KT. et al, 1991-95 Chegini, N. (Co-Invest)	Effect of Polyaromatic Compounds on Placental Proteins. NIH ESO 4435, Direct Cost $573,359.00
Chegini N. (PI) 1991-1993	Peritoneal wound healing: Implication in inflammatory reaction. Regent Medical, Ltd. Direct Cost $122,410.00
Chegini, N. (PI) 1990-91	Eicosanoids, Growth Factors and Wound Healing. Division of Sponsored Research, University of Florida $14,900.00
Chegini N. (PI) 1989-90	Eicosanoids and Growth Factors Action in Uterus. Division of Sponsored Research, University of Florida $10,000.00
Chegini N. (PI) 1988-1989	Immunocytochemical localization of eicosanoids synthesizing enzymes in bovine corpora lutea. Medical School Research Committee University of Louisville $5,500.00
Rao, C.V (PI), 1985-88 Chegini N. (Co-Invest)	Human Choriogonadotropin (hCG) Action in Bovine Luteal Tissue. HD-14697, Direct Cost $175,210

Societies Membership

The Institute of Biology (Fellow)
The Society for Gynecologic Investigation
Society for Study or Reproduction

Wound Healing Society

Editorial Board Membership

1. Frontiers in Bioscience (1997-present)
2. Endocrine (1996-2000)
3. Biology of Reproduction (2003-2004)

Ad hoc Reviewer for Scientific Journals

1. Am J Obstet Gynecol
2. Am J Physiol
3. Adolescent & Pediatric Gynecology
4. Biology of Reproduction
5. British J Cancer
6. British J of OB/GYN
7. Comparative Biochemistry and Physiology
8. Endocrine
9. Endocrinology
10. Eur. J. Endocrinology
11. Fertility & Sterility
12. Human Reproduction
13. Cancer Research
14. Gynecology Oncology
15. J. Clinical Endocrinology and Metabolism
16. J Histochemistry Cytochemistry
17. J Soc Gynocol Invest.
18. Kideny International
19. Molecular & Cell Biochemistry
20. Molecular Cellular Endocrinology
21. Molecular Human Reproduction
22. Obstetrics & Gynecology
23. Regulatory Peptides
24. Tissue and Cell
25. Wound Repair and Regeneration
26. Scientific Program Committee, Society for Gynecological Investigation (1997, 1998,1999, 2001)
27. Chairman; Growth factors and peritoneum; International Congress Peritoneal Tissue Repair. The 4th Peritoneum and Peritoneal Access Meeting. Gothenburg, Sweden, Sept, 16-19, 1997.
28. Moderator; 45th and 48th Annual Meetings of the Society for Gynecological Investigation (1998, 2002).

Ad hoc Reviewer for National and International Granting Organizations

1. March of Dimes
2. Birthright/Royal College of OB/GYN, England
3. United States Department of Agriculture, USDA
4. Medical Research Council of Canada
5. National Sciences and Engineering Research Council of Canada

6. Dutch Cancer Society
7. Belgian Research Council
8. National Institute of Health. REN study section
9. The Welcome Trust, England
10. University Research and Strategic Investments Branch, Alberta Innovation and Science.
11. Michael Smith Foundation for Health Research
12. Philip Morris External Research Program

Administrative Appointment and Committee Member

1. University of Florida Senate representing College of Medicine, 1998-1999, 2001-2003
2. Reviewing committee for University undergraduate Scholarly Research program.
3. Collage of Medicine reviewing committee, Medical Guild, 1999.
4. Chair and Member of Collage of Medicine reviewing committee, Medical Student Research, 2000, 2001, 2002, 2003.
5. Reviewing committee, College of Medicine Research Fund. 2000-2003.
6. Applebaum Visiting Professorship selection committee member, 2000,2001,2002,2003.

Consultant and Advisory Board Membership

1. Focal Inc. Lexington MA, 1994.
2. BioHybrid, Shrewsbury, MA. 2000.
3. Amgen, Inc. Thousand Oak CA, 2000.
4. Genentech, San Fransisco CA, 2000.
5. Regent Medical Allergy Research Group advisory Board Member. Norcross, GA. 1996-present.
6. Federal Drug Administration (FDA), Obstetrics and Gynecology Devices Panel of the Medical Advisory Committee. 1997-2005.

Honors, Awards and Presentations

Honors

1. Cited in the American Men and Women of Science
2. Cited in Who is Who in Science and Engineering, 1993
3. Cited in Who is Who in the World, 1996
4. Cited in Who is Who in America, 1998
5. Certified as Chartered Biologist by the Institute of Biology, 1981
6 Nominee, Dept. of OB/GYN, Univ of FL for Clinical Science Faculty Research Award, 1991
7 Nominee, Dept. of OB/GYN, Univ of FL for Clinical Science Faculty Research Award, 1992.
7. Nominee, Dept. of OB/GYN, Univ of FL for Clinical Science Faculty Research Award, 1995
8. Nominee, Dept. of OB/GYN, Univ of FL for Clinical Science Faculty Research Award, 2003

Awards

1. Traveling awards, University of Louisville, School of Medicine Research Committee continuously from 1982-1988.
2. Endocrine Society Traveling Award, 1984.
3. International Endocrine Society Traveling Award - 1984, Canada.
4. International Endocrine Society Traveling Award - 1988, Japan.
5. Best scientific presentation award, American College of Gynecology, 1988.

6. Best 3rd year resident research project, presented by Gregory A. Miller, M.D., May 1992
7. Residents research advisory award, Department of OB/GYN, University of Florida, 1992
8. Society for Gynecological Investigation Medical Student Stipend for Research in Reproduction, 1998.
9. American Society for Reproductive Medicine, 1998 Prize paper, Society of Reproductive Surgeons. Comparative analysis of matrix metalloproteinase (MMP-1), tissue inhibitor of MMPs (TIMP-1) and MMP-1/TIMP-1 complex expression in intraperitoneal environment and their relation to adhesion development.
10. American Society for Reproductive Medicine, 56th Annual Meeting, San Diego CA, October 21-26, 2000, 3rd place Prize poster. Alteration of transforming growth factor beta expression and autocrine/paracrine actions in leiomyoma smooth muscle cells by gonadotropin releasing hormone analogue (GnRHa), anti-estrogen and anti-progestins. Abst. # p487.
11. Florida Universities Cancer Society Meeting, Feb, 2001, 1st place Prize poster for basic Science presentation. Ripley D and Chegini N. The expression and action of GM-CSF in endometrial cancer.
12. Society for Gynecological Investigation, 51st Annual Meeting, Huoston TX, March, 2004. Traveling award for the presentation of the Abst. Ding L, Luo X and **Chegini N**. (2004) The expression of IL-13 and IL-15 in leiomyoma and myometrium and their influence on TGF-b and proteases expression in leiomyoma and myometrial smooth muscle cells and SKLM, leiomyosarcoma cell line.
13. University of Florida Research Foundation Professorship Award, 2004

Presentations (Invited Speaker).

1. University of Louisville Women's Club, 1982.
2. University of Louisville, Department of OB/GYN Grand Rounds, 1983.
3. Jefferson County High School Administration/Teachers Conference, Louisville, KY, 1983.
4. International Baccalaureate Biology Class of Mr. Ed Farrar (Kentucky Science Teacher of the Year), Atherton High School, Louisville, KY, 1988.
5. Grand Rounds; Department of OB/GYN, University of Florida, Gainesville, FL, 1988. Cellular distribution of eicosanoids enzymes and eicosanoids receptors in the ovary.
6. Scientific Merit of the work, at the 7th Ovarian Workshop, Tacoma, Washington 1988.
7. University of Florida Department of OB/GYN, Grand Rounds, February 1989, Lipoxygenase expression in human reproductive tract tissues.
8. University of Florida Interdisciplinary Reproductive Biology Seminar, Oct. 1989, Growth factors expression in human reproductive tract tissues.
9. University of Florida Department of OB/GYN Grand Rounds, January 1990, The expression and action of growth factors in human uterus.
10 University of Florida, Jacksonville, FL. Dept. of OB/GYN Grand Rounds 1990, Cellular localization of eicosanoids enzymes and eicosanoids receptors in human reproductive tissues.
10. University of Florida "Update In OB/GYN" postgraduate course Orlando FL. March 1991, The role of growth factors in human reproduction.
11. National Institute of Health (NICHD), workshop "Pathogenesis of endometriosis", Sept. 1991.
12. University of Florida Interdisciplinary Reproductive Biology Seminar, Feb. 1994, Growth factor expression in human fallopian tubes.
13. University of Calgary, Dept of Biological Sciences, Canada, March 1994, Growth factor and

Cytokine expression in Human Reproductive Tract Tissues.

14. University of Alberta, Dept of Plastic Surgery, Edmonton, Canada, March 1994. Growth factors and Cytokines in Reproductive Tract Tissues and associated Disorders.
15. University of Florida, Dept of Anatomy and Cell Biology, April, 1994.
16. Wound Healing Society Fifth Annual Meeting, Minneapolis, MN, April, 1995. Peritoneal Adhesion as a model of fibrosis.
17. The University of Kansas School of Medicine-Wichita, Dept. of OB/GYN, Center for Reproductive Medicine, at Wesley Medical Center, June, 1995, Role of TGF-β and GM-CSF in Human Uterus.
18. Symposium on Adhesions: Pathogenesis and Prevention. Gothenburg, Sweden, January, 1996. The role of growth factors in peritoneal healing.
19. The 3rd International Congress on Pelvic Surgery and Adhesion Prevention, San Diego, California, February 1996. Postsurgical peritoneal repair.
20. Genzyme corporation, Boston: Molecular mechanism of peritoneal repair. 1996.
21. Symposium on Post-surgical Adhesions: Linking business and science to further the development of adhesion prevention devices. June 1997. Cellular and molecular events during postsurgical repair. Sponsored by National Managed Care Congress.
22. Karalinska Institute, Department of OB/GYN, Karalinska Hospital, Sweden, 1997. The role of growth factors in leiomyomas.
23. International Congress Peritoneal Tissue Repair.The 4th Peritoneum and Peritoneal Access Meeting. Gothenberg, Sweden, Sept 16-19, 1997. Growth factors and peritoneal adhesion formation.
24. Fibrogen Inc: San Francisco CA. Cellular and molecular mechanism of post-surgical adhesion formation. October 1998.
25. Gliatech Inc: Cleveland, OH. Molecular mechanism of post-surgical adhesions. April 1999.
26. University of Florida Interdisciplinary Reproductive Biology Seminar. Uterine lieomyoma GnRH and GnRH receptor and mechanism of actions. Sept, 1999.
27. 4th International Conference on Postoperative Healing and Adhesions, Fort Lauderdale Fl, Oct. 1999. The role of growth factors in peritoneal repair and adhesion formation.
28. 4th International Conference on Postoperative Healing and Adhesions, Fort Lauderdale Fl, Oct. 1999. The role of matrix metalloproteinases and inhibitors in peritoneal repair and adhesion formation.
29. BioHybrid, Inc., Shrewsbury, MA. Peritoneal tissue fibrosis, Sept, 2000.
31. University of Florida, Dept of Medicine Tissue fibrosis: implication of cytokines and proteases December, 2000.
32. Genentech, Endocrine Divesion "Tissue fibrosis: implication of TGF-β and proteases December, 2000.
33. ISIS Pharmaceutical "Tissue fibrosis", February, 2001.
34. Wayne State University, Michigan, Dept of OB/GYN: Implication of TGF-b and proteases in adhesion formation, March, 2001
35. Wayne State University, Michigan, Dept of OB/GYN: Ground Round: Implication of growth factors in leiomyoma growth and GnRHa-induced regression, March 2001.
36. Intedisplinary Reproductive Biology, University of Florida, The Th1/Th2 cytokines Dichotomy, December 2001.
37. 11th World Congress on Human Reproduction, Montreal Canada, June 2002. Growth factors in

peritoneal tissue fibrosis.

38. American Society for Reproductive Medicine Annual Meeting October, 2002. Adhesion Prevention: The Standard of Care.
39. Regent Medical Sponsored Conference on latex proteins-associated complications. November 2002, Harvard Medical School. Latex Proteins and pro-inflammatory cytokines: Implication in Peritoneal Adhesion.
40. University of Florida, Dept OB/GYN. April 2003. Proteases in health and disease.
41. National Institute of Health (NICHD). June 3003. TGF-b/Ovarian Steroids/GnRH Receptor Signaling: Implication in Gynecological Disorders

Medical Student, Resident and Fellow Training University of Louisville

Reproductive Endocrinology and Gynecologic Surgery Fellows:

Eugene Stoelk, M.D.
Dwight Bailey-Pridham, M.D.
Donald L. Hay, M.D.
Steven A. Metz, M.D.

Medical Student, Resident and Fellow Training University of Florida:

Undergraduate and Graduate Students

Jenny McElhose. Department of Zoology, Summer A/B Course 1991
Heather D. Simpson (Stony Brooks, New York), U.S. Dept. of Education Funded Summer Research Apprenticeship Program 1995
Kerisea McPherson (University of South Florida) Summer Research Apprenticeship Program 1995.
Rebecca Blum, Department of Zoology, January-May 1997.
Alpa Patel, Department of Microbiology, January-May 1998.
Maribeth Buie, IDP graduate student, October-December 1998.
Marisa Roberts, M.Sc Student 1999-2001.
RongXai Li, M.S. Student 2003-present

Graduate Teaching

Department of Anatomy and Cell Biology, University of Florida, Advance Cell Biology, April 1994; Course Director, Dr. Chris West.
Department of Physiology, University of Florida, Advance Endocrinology, November 1996. Course Director; Dr. Charles Wood.
Interdisplinary Graduate Program (IDP), Collage of Medicine, discussion group, 1997, 1998, 2001, 2002, 2003.

Supervisory Committee Member for Ph.D. Student.

Eric Schmitt, Dairy Science (W. W. Thatcher) 1994-96.
Tomislav Mordic, Dairy Science (Frank A. Simmen) 1994-97.
Thais Diaz, Dairy Science (W. W. Thatcher) 1994-98.
Alice de Moraes, Dairy Science (P. Hansen) 1994-98.
Nigh Shi, Department of Medicinal Chemistry (Dona Wieler), 1996-99.
Glenn P. Ladwig, Department of Microbiology and Immunology (Greg Schultz), 1999-2000.
Margaret Hillier, Department of pathophysiology, College of Vet. Sciences (Mary Brown), 1999-

Michelle McGarry, Department of Pharmacology and Therapeutic (Kathleen Shiverick), 1999-2001.
Bilby Todd, Dairy Science (W. W. Thatcher) 2002-present
John Azeke, Biomedical Engineering (E. Goldberg) 2002-present
Jason Blum, IDP, Department of Biochemistry (N. Denslow) 2003-present
Maria B. Padua, Animal Science (P. Hansen) 2003-present

Medical Graduate Students

Narin Arunakul (Senior medical student) research training Feb-March 1990. The effect of epidermal growth factor on human endometrial cells in vitro.

Judy S. Simms (Senior medical student) research training June-July 1990. Localization of growth factors and receptors in endometriosis implants. Data was presented at the American Collage of OB/GYN Annual Meeting, 1991.

Sharrol Barnes, (Senior medical student) research training May-June 1991. The effect of local infection on wound healing and growth factor expression. Data generated from her work was presented in the joint meeting of the European and the American Wound healing Society 1993.

Caroline Cox (Senior medical student) research training November 1991. Immunolocalization of GM-CSF in human cervical tissues in patients with cervical interepithelial neoplasia.

Erin Connor (Senior medical student) research training, May-June 1992 (Graduated with honors in research 1993). The effect of Benzo(a)pyrin on human placental cell growth and EGF receptor content. The data was presented at Endocrine Society Meeting, in 1993 and published in J. Pharm Toxicology 1995.

Reem Abu-Rustum (Third year medical student) research training, Oct-Dec 1992 (Graduated with honors in research 1993). The effect of transforming growth factor on human endometrial stromal cell protein degradation. Published as part of a manuscript in Endocrinology, 1994.

Sam Myrick (Third year medical student) research training March-May 1993, January-Feb, 1994. Immunolocalization of insulin-like growth factor (IGF), IGF-I receptor and IGF binding protein in human ovarian tissue.

Kristina Kostseos (first year Med student) research training June -August 1999, November-December 2001. Expression of TGF-β and adhesion formation.

Megan Mrstik, (first year Med student) research training June -August 1999. Expression of cytokines in wound healing.

Resident and Postdoctoral Fellows Training at University of Florida

OB/Gyn Residents

Robert S. Egerman, M.D. Feb-March 1990. Growth factors and lichen sclerosis. (Data was presented at Resident Research day).

Brian E. Bass, M.D. Feb-March 1991. Estrogenic like effect of EGF on endometrial growth in the rat. (Data was presented at Resident Research day).

Gregory A. Miller, M.D. June-July 1991. Localization of retinoic acid binding protein in human uterine tissue throughout the menstrual cycle. (Data presented at The Annual Meeting of American Fertility Society and Resident Research Day).

Gregory A. Miller, M.D. March 1992. The interaction between retinoic acid and epidermal growth factor in human endometrium. (Data presented at Resident Research day and was selected as First place presentation for the Departmental Resident Research Award).

Barbara M. Faber, M.D. June-July 1991, March 1992, March-April, 1993. Immunolocalization of eicosanoids enzymes in human myometrium in normal and failed labor subjects. (Presented at Resident Research day 1991, 1992 and at the Annual Meeting of the Society for Gynecological Investigation in Chicago). Data published in a manuscript in J. Obstet Gynecol 1994.

Shelley J. Chapman, M.D. Feb-March 1992. Colocalization of growth factors in fetoplacental units in normal and chorioamnionitis patients.

Judy S. Simms, M.D. July-August 1992. Immunolocalization of epidermal growth factor, transforming growth factor alpha and their receptors in peritoneal adhesion (Data was presented at Resident Research day, and was published in two manuscripts in Obstet Gynecol and Am J Obstet Gynecol 1994).

Caroline Cox M.D. Oct-Nov 1993. Immunolocalization of GM-CSF in human Cervix.

Amy Boardman. M.D. June-July 1993. Immunolocalization of growth factors in porcine oviduct. In collaboration with Dr. Buhi.

Anil Sood, M.D. June-July 1994. Immunolocalization of growth factors in human feto-placental tissues in patients with chorioamnionitis.

Brenda Shoup, M.D. March-April 2001. Cytokine and ovarian cancer

Eric Levens, M.D., May 2004, Gene expression profile in leiomyoma

Postdoctoral Fellows		**Current Position**
Michael J. Rossi, Ph.D.	1989-1992	(Associate Professor, University of New Heaven)
Xin-Min Tang, M.D.	1991-1996	(Research Scientist, University of South Florida, Resident Medicine USF)
Yong Zhao, M.D.	1992-1995	
Thea L. Pfeifer, M.D.	1991-1993	(Pediatric Endocrine Fellow, Private practice)

Hue Rong, M.D.	1993-1995	(Women Health Center, Nashville TN)
Qingchuan Dou, Ph.D.	1994-1997	
Yong Zhao, M.D.	1997-1998	(Resident in Pathology, Vanderbilt University, Fellow, University of Iowa and Virginia)
Chunfeng Ma, M.D., Ph.D.	1997-2000	(Research Fellow, Dept of Medicine, John Hopkins University)
Yengshi Gao, M.D.	1998-1999	
Baong Sui, Ph.D.	2000-2001	(Research Scientist, University of Maryland)
Jing-Xia Xu M.D.	2001-2002	(Jackson laboratory Bar Harbor)
Xiaoping Luo M.D.	2001-present	
Li. Ding M.D	2002-present	

National and International Collaboration.
Ahmad R Safa, Ph.D. Department of Medicine, The University of Chicago; Aziz Ghahary, Ph.D. Department of Surgery, University of Alberta, Edmonton Canada, Leslie Gold, Ph.D., Department of Pathology, University of New York, Hamid Habibi, Ph.D., Department of Zoology, University of Calgary, Canada, Lena Holmdahl, M.D., Ph.D., Department of Surgery, Gothenburg University, Gothenburg, Sweden. Michael Diamond, Wayne State University, Detroit, Michigan, Barbara Faber, M.D. and David Archer, M.D. The Jones Institute For Reproductive Medicine, Norfolk VA.

Citation analysis
Science Citation Index list 3,766 cumulative citations to Dr. Chegini's refereed publications in August 2003: 19 publications with >50 citations, 4 publications with >100 citations, 2 publications with >150 citations.

PEER REVIEWED RESEARCH PUBLICATIONS

1. **Chegini N**, Aleporou V, Bell G, Hilder VA and Maclean N (1979) Production and fate of erythroid cell in anaemic *Xenopus Laevis*. J. Cell Sci. 35:403-415.

2. **Chegini N** and Maclean N (1980) Chromatin organization within nuclear blebs in Leukocytes of *Xenopus Laevis*. Experintia. 38:876.

3. **Chegini N**, Gregory SP, Hilder VA, Pocklington MJ and Maclean N (1981) Structural transition of chromatin in isolated *Xenopus* erythrocyte nuclei. I: The effect of ions. J. Submicroscopic Cytol. 13:291-308.

4. **Chegini N**, Hilder VA, Gregory SP and Maclean N (1981) Structural transition of chromatin in isolated *Xenopus* erythrocyte nuclei. II: Computer-Based image analysis. J. Submicroscopic Cytol. 13:309-320.

5. Safa AR, **Chegini N** and Tseng MT (1983) Influence of mitoxantrone on nucleic acid synthesis on the T-47D breast tumor cell line. J. Cell. Biochem. 22:111-120.

6. **Chegini N**, Safa AR and Tseng MT (1984) Acute effects of mitoxantrone on the template

activity of isolated nuclei from the T-47D human breast tumor cell line. Cancer Letters 21:329-336.

7. **Chegini N**, Rao CV and Carman FR Jr. (1984) Internalization of ^{125}I-human choriogonadotropin in bovine luteal slices: A biochemical study. Exp. Cell. Res. 151:466-482.

8. **Chegini N**, Rao CV and Cobbs G (1984) A quantitative electron microscope autoradiographic study on ^{125}I-human choriogonadotropin internalization in bovine luteal slices. Exp. Cell. Res. 151:483-493.

9. **Chegini N**, Ramani N and Rao CV (1984) Morphological and biochemical characterization of small and large bovine luteal cells during pregnancy. Mol Cell Endocrinol 34:89-112.

10. Rao CV, Carman FR Jr., **Chegini N** and Schultz GS (1984) Binding sites for epidermal growth factor in human fetal membranes. J. Clin. Endocrinol. Metabol. 58:1034-1042.

11. **Chegini N**, Rao CV and Cobbs G (1984) A quantitative electron microscope autoradiographic study on ^{3}H-prostaglandins E_1 and $F_{2\alpha}$ internalization in bovine luteal slices. Mol. Cell. Endocrinol. 38:117-129.

12. Rao CV, Ramani N, **Chegini N**, Stadig BK, Carman FR Jr., Woost PG, Schultz GS and Cook CL (1985) Topography of human placental receptors for epidermal growth factors. J. Biol. Chem. 260:1705-1710.

13. **Chegini N** and Rao CV (1985) Epidermal growth factor binding to human amnion, chorion, decidua and placenta from mid and term pregnancy: Quantitative light microscope autoradiographic studies. J. Clin. Endocrinol. Metabol. 61:529-535.

14. **Chegini N**, Rao CV, Wakim N and Sanfilippo J (1986) Prostaglandin receptors in different cell types of human uterus during proliferative and secretory phases. Prostaglandin, Leukotrienes and Med. 22:129-138.

15. Ramani N, **Chegini N**, Rao CV, Woost PG and Schultz GS (1986) The presence of epidermal growth factor binding sites in the intracellular organelles of term human placenta. J. Cell. Sci. 84:19-40.

16. **Chegini N**, and Rao CV (1986) A quantitative electron microscope autoradiographic study of ^{125}I- epidermal growth factor internalization in term human placenta. J. Cell Sci. 84:41-52.

17. **Chegini N** and Rao CV (1986) Histochemical demonstration of 3β-hydroxy steroid dehydrogenase in cells of amnion, chorion, decidua and placenta from mid and term pregnancy. International Res. Commun. Ser. Med. Sci. (I.R.C.S.) Biochem. 14:986-987.

18. **Chegini N**, Rao CV, Wakim N and Sanfilippo J (1986) Binding of ^{125}I-epidermal growth factor to human uterus. Cell & Tissue Res. 246:231-236.

19. Shahabi NA, **Chegini N** and Wittliff JL (1987) Alterations of MCF-7 human breast cancer cell after prostaglandins PGA_1 and $PGF_{2\alpha}$ treatment. Exp. Cell. Biol. 55:18-27.

20. **Chegini N**, Hay DL, von Fraunhofer JA, Stone K and Masterson BJ (1987) The use of nylon pouch to prevent cellular attachment to implant materials. Biomaterials 8:315-319.

21. **Chegini N** and Rao CV (1987) Dynamics of nuclear associated granules in Bovine luteal cells after treatment in vitro with prostaglandin $F_{2\alpha}$. Endocrinology 121:1870-1878.

22. **Chegini N** and Safa AR (1987) Morphological alterations induced by prostaglandins PGE_1, $PGF_{2\alpha}$ and PGA_1 in MDA-MB-231 and MCF-7 human breast tumor cell lines. Cancer Letters 37:189-197.

23. **Chegini N** and Safa AR (1987) Influence of mitoxantrone on nucleolar function in MDA-MB-231 human breast tumor cell line. Cancer Letters 37:327-336.

24. **Chegini N**, Hay DL, von Fraunhofer JA and Masterson BJ (1988) The effects of bacterial infection on absorbable ligating clips. J. Reprod. Med. 33:25-29.

25. **Chegini N**, Hay DL, von Fraunhofer JA and Masterson BJ (1988) A comparative scanning electron microscopic study on degradation of absorbable ligating clips in vivo and in vitro. J. Biomed. Materials Res. 22:71-79.

26. **Chegini N**, von Fraunhofer JA, Hay DL and Masterson BJ (1988) The tissue reaction to absorbable ligating clips. J. Reprod. Med. 33:187-192.

27. Hay DL, von Fraunhofer JA, **Chegini N** and Masterson BJ (1988) Locking mechanisms strength of absorbable ligating devise. J. Biomed. Materials Res. 22:179-190.

28. **Chegini N** and Rao CV (1988) The presence of Leukotriene C_4 and prostacyclin binding sites in non-pregnant human uterine tissue. J. Clin. Endocrin. Metabol. 66:76-87.

29. **Chegini N** and Rao CV (1988) Increased nuclear bodies in bovine luteal cells following hCG treatment. Biol. Reprod. 38:453-461.

30. **Chegini N** and Rao CV (1988) Quantitative light microscopic autoradiographic study on ^3H-Leukotriene C_4 binding to non-pregnant bovine uterus. Endocrinology 122:1732-1736.

31. **Chegini N** and Rao CV (1988) The presence of Leukotriene C_4 binding sites in bovine corpora lutea of pregnancy. Biol. Reprod. 39:929-935.

32. Metz SA, **Chegini N** and Masterson BJ (1989)In vivo tissue reactivity and degradation of sutures materials: A comparison of Maxon and PDS. J. Gynecol. Surg. 5:37-46.

33. **Chegini N** and Rao CV (1989) The presence of prostacyclin binding sites in nonpregnant bovine uterine tissue. Prostaglandin Leukotrienes & Essential Fatty Acids 38:75-79.

34. Oechsli M, Rao CV and **Chegini N** (1989) Human chorionic gonadotropin increases chromatin solubility in isolated bovine and human luteal nuclei. Biol. Repod. 41:753-760.

35. Metz SA, **Chegini N** and Masterson BJ (1990) In vivo and in vitro degradation of monofilament absorbable sutures. Biomaterials 11:41-45.

36. Reshef E, Lei ZM, Rao CV, Bailey-Pridham DD, **Chegini N** and Luborsky JL (1990) The presence of human chorionic gonadotropin/luteinizing hormone receptors in nonpregnant human uteri. J. Clin. Endocrinol. Metabol. 70:421-430.

37. **Chegini N**, Metz SA and Masterson BJ (1990) Tissue reactivity and degradation patterns of absorbable vascular ligating clips implanted in peritoneum and rectus fascia. J. Biomed. Materials Res. 24:929-937.

38. Bailey-Pridham D, Lei ZM, **Chegini N**, Rao CV, Yussman MA and Cook CL (1990) Light and electron microscope immunocytochemical localization of 5 and 12-lipoxygenase and cyclooxygenase enzymes in human granulosa cells from preovulatory follicles. Prostaglandin Leukotrienes & Essential Fatty Acids 39:231-238.

39. **Chegini N**, Rao CV and Lei ZM (1990) Cellular distribution of prostacyclin synthase and prostacyclin binding sites in bovine corpora lutea of pregnancy. Mol. Cell. Endocrinol. 71:133-140.

40. **Chegini N**, Lei ZM, Rao CV and Bischof P (1991) The presence of pregnancy association plasma protein-A in human corpora lutea: cellular and subcellular distribution and dependency or reproductive states. Biol. Reprod. 44:201-206.

41. **Chegini N**, Lei ZM and Rao CV (1991) Nuclear volume and chromatin pattern changes in bovine luteal cells following treatment with human chorionic gonadotropin, prostaglandins and cyclic AMP. Cell & Tissue Res. 264:453-460.

42. Stoelk E, **Chegini N**, Lei ZM, Rao CV, Bryant-Greenwood G and Sanfilippo J (1991) Immunocytochemical localization of relaxin in human corpora lutea:cellular distribution and dependency on reproductive states. Biol. Reprod. 44:1140-1147.

43. Lei ZM, **Chegini N** and Rao CV (1991) Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol. Reprod. 44:1148-1156.

44. Bibbins PE Jr., Rao CV, Carman FR and **Chegini N** (1991). Role of luteal cell nucleus in the expression of gonadotropin action. J. Endocrin. Invest. 14:391-400.

45. **Chegini N**, Lei ZM, Rao CV and Hansel W (1991) Cellular distribution and cycle phase

dependency of gonadotropin and eicosanoid binding sites in bovine corpora lutea. Biol. Reprod. 45:506-513.

46. Simms JS, **Chegini N**, Williams RS, Rossi AM, and Dunn WA Jr (1991) Identification of epidermal growth factor, transforming growth factor alpha, and epidermal growth factor receptor in surgically induced endometriosis in rat. Obstet. Gynecol. 78:850-857.

47. **Chegini N,** and Williams RS (1992) Immunocytochemical localization of transforming growth factor (TGFs) TGF-α and TGF-β in human ovarian tissue. J. Clin. Endocrinol. Metab. 74:973-980.

48. Williams RS, Rossi AM, **Chegini N** and Schultz GS (1992) Effect of transforming growth factor-beta on postoperative adhesions formation and intact peritoneum. Surg. Res. 52:65-70.

49. Rossi MJ, **Chegini N** and Masterson BJ (1992) Presence of epidermal growth factor, platelet-derived growth factor and their receptors in human myometrial tissue and smooth muscle cells:their action in smooth muscle cell *in vitro*. Endocrinology. 130:1716-1727.

50. **Chegini N**, Rossi MJ and Masterson BJ (1992) Platelet-derived growth factor, epidermal growth factor and EGF and PDGF β receptors in human endometrial tissue: Localization and *in vitro* action. Endocrinology 130:2763-2775.

51. **Chegini N**, and Flanders KC (1992) Presence of transforming growth factor-β (TGF-β1, GF-β2) and their selective localization in human ovarian tissue of various reproductive states. Endocrinology 130:1707-1715.

52. Khaw PT, MacKay SLD, **Chegini N,** and Schultz G (1992) TGF-α autocrine system in human corneal epithelium. Invest. Ophthalmol. Vis. Sci. 33:3302-3306.

53. Schultz GS, Strelow S, Stern GS, **Chegini N**, Grant MB, Galardy RE, Grabelny D, Rowsey JJ, Stonecipher C and Parmley V (1992) Treatment of Alkali-Injured rabbit corneas with a Synthetic inhibitor of matrix metalloproteinases. Invest. Ophthalmol. Vis. Sci. 33:3325-3331.

54. **Chegini N,** Rossi MJ, Schultz GS, Dunn WA Jr. and Masterson BJ (1993) Cellular distribution of EGF, TGF-α and EGF receptor in fascia and peritoneum during healing: An autoradiographic and immunocytochemical study. J. Wound Rep. & Reg. 1:27-40.

55. Grant MB, Mames RN, Fitzgerald EN, Ellis EA, Caballero S, **Chegini N** and Guy J (1993)Insulin-like growth factor induced retinal fibrovascular proliferation: A model for diabetic retinopathy. Annu. New York Acad. Sci. 692:230-242.

56. Humphreys-Beher MG, Brinkley L, Purushotham K, Wang P-L, Nakagawa Y, Dusek D, Kerr M, **Chegini N** and Chan EKL (1993) Characterization of antinuclear autoantibodies present in the serum from non-obese diabetic (NOD) mice. Clin. Immunol. Immunopath. 68:350-356.

57. King JD, Stringer SP, **Chegini N**, Donnelly WH, Cassisi NJ and Schultz GS (1993) Transforming growth factor alpha protein and receptor localization in normal and healing laryngotracheal tissue. Otolaryngol. Head & Neck Surg. 109:915-925.

58. Humphreys-Beher MG, Macauley S, **Chegini N**, Van Setten G, Purushotham K, Stewart C, Wheeler TT and Schultz GS (1993) Characterization of the synthesis and secretion of TGF-α from salivary glands and saliva. Endocrinology 134:963-970.

59. Wilson SE, Schultz GS, **Chegini N**, Weng J and He Y-G (1994) Epidermal growth factor, transforming growth factor alpha, transforming growth factor beta, acidic fibroblast growth factor, basic fibroblast growth factor, and interleukin-1 protein in the cornea. Exp Eye Res 59:63-71.

60. Pfeifer TL and **Chegini N** (1994) Immunohistochemical localization of insulin-like growth factor(IGF-I), IGF-I receptor (IGF-IR) and IGF binding proteins 1-4 in human fallopian tube at various reproductive stages. Biol. Reprod. 50:281-289.

61. **Chegini N**, Zhao Y, and McLean FW (1994) The expression of mRNA and presence of immunoreactive proteins for Epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and EGF/TGF-α receptors and ^{125}I-EGF binding sites in human fallopian tube. Biol. Reprod. 50:1049-1057.

62. Tang X-M, Rossi MJ, Masterson BJ and **Chegini N** (1994) Insulin-like growth factor I (IGF-I), IGF binding proteins and IGF-I receptor in human uterine tissue: Localization and IGF-I action in endometrial stromal and myometrial smooth muscle cells *in vitro*. Biol. Reprod. 50:1113-1125.

63. **Chegini N**, Gold LI, Williams RS and Masterson BJ (1994) Localization of Transforming Growth Factor Beta Isoforms TGF-β1, TGF-β2 and TGF-β3 in Surgically induced Pelvic Adhesion in the rat. Obstet. Gynecol. 83:449-454.

64. **Chegini N**, Gold LI and Williams RS (1994) Localization of Transforming Growth Factor Beta Isoforms TGF-β1, TGF-β2 and TGF-β3 in Surgically-induced Endometriosis in the rat. Obstet. Gynecol. 83:455-461.

65. **Chegini N**, Zhao Y, Williams RS and Flanders KC (1994) Human uterine tissue throughout the menstrual cycle expresses TGF-β1, TGF-β2 TGF-β3 and TGF-type II receptor mRNA and proteins and contain ^{125}I-TGF-β1 binding sites. Endocrinology 135:439-449.

66. Tang X-M, Zhao Y, Rossi MJ, Abu-Rustum RS, Ksander GA and **Chegini N** (1994) Expression of TGF-β isoforms and TGF-β type II receptor mRNA and protein, and the effect of TGF-βs on endometrial stromal cells growth and protein degradation *in vitro*. Endocrinology 135:450-459.

67. **Chegini N**, Simms JS, Williams RS and Masterson BJ (1994) Identification of epidermal

growth factor, transforming growth factor alpha, and epidermal growth factor receptor in surgically-induced pelvic adhesion disease in the rat an intraperitoneal adhesion in human. Am. J. Obstet. Gynecol. 175:321-328.

68. Kerr M, Fischer JE, Purushotham KR, Gao D, Nakagawa Y, Maeda N, Ghanta V, Hiromato R, **Chegini N** and Humphreys-Beher MG (1994) Characterization of the synthesis and Expression of the GTA-kinase (p58) from transformed and normal rodent cells. Biochem Biophys. Acta. 1218:375-387.

69. Schultz GS, Khaw PT, Oxford K, Macauley S, Van Setten G and **Chegini N** (1994) Growth factors and ocular wound healing. Eye 8:184-187.

70. Zhao Y and **Chegini N** (1994) Human fallopian tube expresses granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors and contain the immunoreactive GM-CSF protein. J. Clin. Endocrinol. Metab. 79:662-665.

71. Zhao Y, **Chegini N** and Flanders KC (1994) Human Fallopian Tube Expresses Transforming Growth Factor β (TGF-β) Isoforms and TGF-β type I-III receptor messenger ribonucleic acids and proteins and Contain ^{125}I-TGF-β1 binding sites. J. Clin. Endocrinol. Metab. 79:1177-1184.

72. Tang X-M, **Chegini N**, Rossi MJ, Fay MF, and Masterson BJ (1994) The effect of surgical glove powder on proliferation of human skin fibroblasts and monocyte/macrophage. J. Gynecol. Surg. 10:139-150.

73. Juneja SC, Pfeifer TL, Williams RS and **Chegini N** (1994) Neem oil inhibits the 2-cell embryo development, trophectoderm attachment and proliferation *in vitro*. J. Assisted. Reprod. Genetics. 11;419-427.

74. Kerr M, Lee A, Wang P-L, Purushotham K, **Chegini N,** Yamamoto H and Humphreys-Beher MG (1994) Detection of insulin, insulin-like growth factors I and II in saliva and synthesis in the salivary glands of mice. Effect of type I diabetes mellitus. Biochem. Pharmacol.49,1521-1531.

75. Juneja SC, Pfeifer TL, Tang XM, Williams RS and **Chegini N** (1995) Modulation of mouse sperm-egg interaction, early embryonic development and trophoblastic outgrowth by activated and unactivated macrophages. Endocrine 3:69-79.

76. Tang X-M, and **Chegini N** (1995) Human fallopian tube expresses mRNA and protein for relaxin and contain specific relaxin receptor. Biol. Reprod 52:1343-1349.

77. Zhao Y, Rong H and **Chegini N** (1995) The expression and selective cellular localization of granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors mRNA and protein in human ovarian tissue. Biol Reprod.53:923-930.

78. Zhang L, Connor EE, **Chegini N** and Shiverick KT (1995) Benzo(a)Pyrene modulates

epidermal growth factor receptors, cell proliferation and secretion of human chorionic gonadotropin in human placental cell lines. Biochem Pharmcol. 50:1171-1180.

79. Tang X-M, **Chegini N,** Fay MF and Masterson BJ (1995) Surgical glove powders differentially modulate macrophage and lymphocyte-derived cytokines and eicosanoids production *in vitro*. Wound Rep & Reg. 3:518-526.

80. Dou, Q, Zhao Y, Tarnuzzer RW, Rong H, Williams RS, Schultz GS, and **Chegini N**. (1996) Suppression of TGF-βs and TGF-β receptors mRNA and protein expression in leiomyomata in women receiving gonadotropin releasing hormone agonist therapy. J Clin Endocrinol Metab 81:3222-3230.

81. **Chegini N**, Rong H, Dou Q, Kipersztok S, and Williams RS (1996).Gonadotropin releasing hormone (GnRH) and GnRH receptor gene expression in human myometrial and leiomyomta and the direct action of GnRH analogs on myometrial smooth muscle cells interaction with ovarian steroids in vitro J Clin Endocrinol Metab. 81:3215-3221

82. Faber BM, Metz SA, and **Chegini N** (1996) Immunolocalization of eicosanoid enzymes and growth factors in human myometrium and fetoplacental tissues in failed labor inductions. Obstet Gynecol. 88:174-179

83. Juneja S, **Chegini N,** Williams RS and Ksander G (1996) Ovarian intrabursal administration of recombinant TGF-β1 inhabits follicle rupture in gonadotropin-primed mouse. Biol Reprod 55:1444-1451.

84. Juneja S, Williams RS, Farooq A, **Chegini N** (1996). Contraception potential of Neem oil: Effect on pregnancy success in the mouse. J Assisted Reprod Gent 13:578-585.

85. Tang X-M, Dou Q, Zhao Y, Davis J, McLean WF, and **Chegini N** (1997) Expression mRNA and protein for TGF-β isoforms and TGF-β type I-III receptor in human myometrial smooth muscle cells and their effect on DNA synthesis and protein degradation *in vitro*. Mol Hum Reprod 3:233-240.

86. Rong H, Tang X-M, Zhao Y, Juneja SC, Fay MF, Williams RS, and **Chegini N.** (1997) Peritoneal exposure to surgical glove powders differentially modulate the inflammatory and immune related cytokines production during surgically-induced adhesion formation. Wound Rep & Reg 5:89-96.

87. **Chegini N,** Rossi MJ and Holmdahl LE (1997) Cellular distribution of 5-lipoxygenase and leukotrienes receptors in postsurgical peritoneal wound repair. Wound Res & Reg 5:235-242.

88. Yamamoto H, Ishibashi K, Nakagawa Y, Maeda N, Zeng T, Robinson CP, Oxford GE, **Chegini N,** and Humphreys-Beher MG (1997) Detection of alteration in the levels of neuropeptides and salivary gland responses in the non-obese diabetic mouse model for autoimmune sialoadenitis. Scan J Immunol 45:55-61.

89. Dou Q, Williams RS and **Chegini N** (1997) Inhibition of transforming growth factor-β1 alters the growth, anchor-dependent cell aggregation and integrins mRNA expression in human promonocytes: Implications for endometriosis and peritoneal adhesion formation. Mol Hum Reprod. 3:383-391.

90. Ghassemifar MR, Tarnuzzer RW, **Chegini N**, Tarpila E, Schultz GS, Franzen LE (1997). Expression of alpha smooth muscle actin, TGF-β1 and TGF-β type II receptor during connective tissue construction. In Vitro Cell Dev Biol 33:622-627.

91. Dou Q, Tarnuzzer RW, Williams RS, Schultz GS, and **Chegini N** (1997) Differential expression of matrix metalloproteinases and their tissue inhibitors in leiomyomata: A mechanism for gonadotropin releasing hormone agonist induced tumour regression. Mol Hum Reprod. 3:1005-1014.

92. Holmdahl L and **Chegini N** (1997). Endotoxin and particulate matter on surgical gloves. J. Long term effects of Medical Implants. 7:225-234.

93. Macauley SP, Tarnuzzer RW, Schultz GS, **Chegini N,** Oxford GE, and Humphreys-Beher MG (1997). Identification of coordinated expression of extracellular matrix genes during mouse submandibular gland development by quantitative competitive RT-PCR. Arch Oral Biol. 42:443-445.

94. Kothapalli R, Buyuksal I, Wu SQ, **Chegini N**, Tabibzadeh S (1997). Detection of *ebaf*, a novel human gene of the transforming growth factor β superfamily. J Clin Invest 99; 2342-2350.

95. Dou Q, Williams RS and **Chegini N** (1999) Expression of integrin mRNA in human endometrium. A quantitative RT-PCR study. Fertil Steril 71:347-353.

96. **Chegini N**, Tang XM and Dou Q (1999) The expression, action and regulation of granulocyte macrophage-colony stimulating factor in human endometrial epithelial and stromal cells. Mol Hum Reprod 5:459-466.

97. deMoraes AAS, Paula-Lopes FF, **Chegini N** and Hansen PJ (1999). Immunohistochemical localization of granulocyte macrophage-colony stimulating factor (GM-CSF) in the bovine reproductive tract. J Reprod Immunol. 76:135-145

98. **Chegini N** and Rong H (1999) Peritoneal exposure to surgical glove powders modulate the eicosanoids production during surgically-induced adhesion formation. Eur J Surg., 171, 698-704.

99. **Chegini N,** Dou Q, Wiliams RS (1999). An inverse relation between TNF-α and TNF-α receptor mRNA expression in human endometrium. Am J Reprod Immunol. 42;297-302.

100. **Chegini N**, Rong H, Bennett B and Stone IK (1999) Peritoneal fluid levels of cytokine and

eicosaniod and their relation to the incidence of peritoneal adhesion. J Soc Gynecol Invest 6. 154-157.

101. Zhao Y and **Chegini N** (1999) The expression of granulocyte macrophage-colony stimulating factor (GM-CSF), GM-CSF α and β receptors in human uterine tissue. Am J Reprod Immunol 42: 303-311.

102. **Chegini N,** Ma C, Davis J, Duff P and Rosa C (1999) Differential expression of transforming growth factor β1 (TGF-β1) and TGF-β receptors in myometrium of women with failed labor-induction, no labo and preterm labor. J Soc Gynecol Invest. 6:258-263.

103. Ma C and **Chegini N** (1999) Matrix metalloproteinases and tissue inhibitor of MMPs (TIMPs): Expression in human myometrial smooth muscle cell and regulation by transforming growth factor β1. Mol Hum Repord. 5:950-954

104. **Chegini C**, Tang XM and Ma C (1999). Regulation of transforming growth factor β1 expression by granulocyte macrophage-colony stimulating factor (GM-CSF) in leiomyomas and myometrial smooth muscle cells. J Clin Endocrinol Metabol. 84:4138-4143.

105. Ma C, Tarnuzzer RW and **Chegini N** (1999) Modulation of matrix metalloproteinases and tissue inhibitor of MMPs (TIMPs) in human mesothelial cells by transforming growth factor β1. Wound Res & Reg. 7: 477-485.

106. Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP (1999). Collagen I and III production by human peritoneal mesothelial cells in response to hypoxia and TGF-β1 treatments. Wound Rep & Regen.7:504-510.

107. Ghellai AM, Stucchi AF, **Chegini N**, Ma C, Kaseta JM, Andry CD, Burns JW, Skinner KC and Becker JM (2000). The role of transforming growth factor beta-1 (TGF-β1) in Peritonitis-induced adhesions. J Gastrointest Surg;4:316-323.

108. Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP and the PHAMUS group (2000). Transforming growth factor beta isoforms (TGF-βs) production by human peritoneal mesothelial cells in response to hypoxia treatments. Am J Reprod Immunol. 43;285-291.

109. Holmdahl L, Blum R, **Chegini N**, Iversson ML, Bergstrom M (2000). Coagulation and fibrinolysis in peritoneal tissue. Wound Rep & Regen. In press

110. Flak P, Ma C, **Chegini N** and Holmdahl L (2000) Differential regulation of mesothelial cells fibrinolysis by transforming growth factor beta 1. Scand J Clin Lab Invest; 60:439-448.

111. Koenig JM and **Chegini N**. (2000). Enhanced expression of Fas-associated proteins in fetal membranes, decidual and placents of pregnancy-induced hypertension. Am J Reprod. Immunol. 44; 347-349.

112. Calhoun DA, **Chegini N**, Polliotti BM, Du Y, Gersting JA, Miller RK, Christensen RD. (2001). Granulocyte colony-stimulating factor in preterm and term pregnancy, parturation and intra-amniotic infection. Am J Obstet Gynecol. 97:229-234.

113. Holmdahl L, Kotseos K, Bergstrom M, Iversson ML, Falk P, and **Chegini N** (2001). Overproduction of TGF-β is associated with adhesion formation and fibrinolytic impairment. Surgery.129:626-632

114. Chegini N, **K Kotseos**, C Ma, B Bennett, RS Williams, MP Diamond, L Holmdahl, K Skinner (2001) Differentail expression of integrin av and b3 in serosal tissue of human intraperitoneal organs and adhesions. Fertil. Steril. 75:791-796.

115. Ripley D,Tang XM, Ma C, Zhao Y and **Chegini N**. (2001). The Expression and action of granulocyte macrophage-colony stimulating factor (GM-CSF) in endometrial carcinoma. Gynecologic Oncology. 81:301-309.

116. **Chegini N**. K Kotseos, Y Zhao, C Ma, F McLean, MP Diamond, L Holmdahl, J Burns (2001) The expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in serosal tissue of intraperitoneal organs and adhesions. Fertil Steril 76: 1212-1219.

117. **Chegini N,** Kotsesos K, Bennett B, McLean FW, Diamond MP, Holmdahl L, Burns J (2001) Matrix Metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in peritoneal fluids and sera, and correlation with peritoneal adhesions. Fertil Steril 76:1207-1211

118. **Chegini N,** K Kotseos, Y Zhao, B Bennett, FW, McLean, MP Diamond, L Holmdahl, J Burns (2001) Differential expression of transforming growth factor β in serosal of tissue of human intraperitoneal organs and peritoneal adhesions. Hum. Reprod. 16:1291-1300.

118. Faber, BM, **Chegini N,** Mahony MC, and Coddington CC (2001) Macrophage secretory products impair sperm Zona Pellucida binding: a cause of endometriosis associated infertility. Obstet Gynecol 98:668-673.

119. Svinarich DM, Zaher FM, Holmdahl L, **Chegini N**, Gonik B, Diamond MP (2001). Adhesion development and the expression of endothelial nitric oxide synthase. Infect Dis Obstet Gynecol. 9:113-116.

120. Umekawa T, **Chegini N** and Khan SR (2002). Oxalate ions and calcium oxalate crystals stimulate MCP-1 expression by renal epithelial cells. Kidney Int.61:105-112.

121. Dame JB, Chegini N, Christensen RD, Juul SE (2002). The effect of interleukin-1beta (IL-1b) and tumor necrosis factor-alpha (TNF-a) on granulocyte macrophage-colony stimulating factor (GM-CSF) production by neuronal precursor cells. Eur Cytokine Netw. 2002;13:128-133.

122. **Chegini N,** Y Zhao, K Kotseos, C Ma, B Bennett, MP Diamond, L Holmdahl, K Skinner (2002) The expression of matrix metalloproteinase (MMP-3) and tissue inhibitor of MMP

(TIMP-2) in serosal of intraperitoneal tissues and adhesions in human. Brit J Obstet Gynecol 1041-1049.

123. Chegini N, Ma, C., Roberts M., Williams RS, and BA Ripps (2002). Differential expression of interlukines (IL) IL-13 and IL-15 in human endometrium from normal fertile and recurrent spontaneous abortion. J Reprod Immunol 56, 93-110.

124. **Chegini N,** Ma C, Tang XM, Williams RS (2002). Effects of GnRH analogues, 'add-back' steroid therapy, antiestrogen and antiprogestins on leiomyoma and myometrial smooth muscle cell growth and transforming growth factor-beta expression. Mol Hum Reprod. 8:1071-1078.

125. **Chegini N** and Kornberg L. (2003). Gonadotropin releasing hormone analogues (GnRHa) Therapy alters signal transduction pathways involving MAP and focal adhesion kinases in leiomyoma. J Soc Gynecol Invest. 10: 21-26.

126. Umekawa T, **Chegini N**, Khan SR (2003) Increased expression of monocyte chemoattractant Protein –1 (MCP-1) by renal epithelial cells in culture on exposure to calcium oxalate, phosphate and urate crystals. Nephrol Dial Transplant.18:664-669.

127. **Chegini N**, Roberts M., and Ripps BA (2003). Differential expression of interlukines (IL) IL-13 and IL-15 in endometrial implants and peritoneal fluids of women with endometriosis Am. J Reprod Immunol. 49:75-83.

128. Chegini N, Verala J, Luo X, Xu J and Williams RS. (2003) Gene expression profile of leiomyoma and myometrium and the effect of gonadotropin releasing hormone analogue (GnRHa) therapy. J Soc Gynecol Invest. 10:161-171.

129. Rhoton-Vlasak A, Gleich JG, Bischof P, and **Chegini N.** (2003) Localization and cellular distribution of pregnancy-associated plasma protein–A and major basic protein in human ovary and corpora lutea throughout the menstrual cycle. Fertil Steril. 2003;79:1149-53.

130. Xu J, Luo X and **Chegini** N. (2003). Differential expression, regulation and activation of Smads, TGF-b signal Ttansduction pathway, in leiomyoma and myometrial smooth muscle cells and alteration by gonadotropin releasing hormone analogue (GnRHa). J Clin Endocrin Metabol. 83:1350-1361.

131. Mrstik M, Kotseos M, Ma C and **Chegini N.** (2003). Increased expression of interferon-gamma inducible protein-10 (IP-10) mRNA during surgically-induced peritoneal injury. Wound Rep and Regen.11:120-126.

132. Tunuguntla R, Ripley D, Sang Q-X-A and **Chegini N.** (2003) The Expression of matrix matelloproteinase (MMP-26) and tissue inhibitor of MMPs (TIMP)-3 and –4 in benign endometrium and endometrial cancer. Gynecol. Oncol 89:453-439.

133. **Chegini N**, Rhoton-Vlasak A and Williams RS (2003) Matrix metalloproteinase-26 and tissue

inhibitor of MMP (TIMP-4) expression in endometrium throughout the normal menstrual cycle and alteration in norplant users experiencing irregular uterine bleeding. Fertil Steril. 80:564-570.

134. Luo, X, Xu, J and **Chegini N**. (2003) The expression of Smads in human endometrial epithelial and stromal cells and regulation by TGF-b. J Clin Endocrinol Metabol. 88:4967-4976.

135. Luo X, Xu J and **Chegini N**. (2003) Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smads in human endometrial epithelial and stromal cells. J Reprod Biol Endocrinol 1; 1-13.

136. **Chegini N,** Ding L, Luo X and Ripley D (2003) Alteration of TGF-β receptors and Smads expression in leiomyoma and myometrium following Gonadotropin Releasing Hormone (GnRHa) therapy. Mol Cell Endocrinol. 209:9-16.

137. **Chegini N**, Ripley D, Shoup B and Majewski A.(2004) Differential expression of interleukins (IL) IL-13 and IL-15 in normal ovarian tissue and ovarian carcinomas. Gynecol. Oncology. 92:761-768.

138. Ripley D, Tunuguntla R, Sui L, **Chegini N.** (2004) The expression of matrix matelloproteinase (MMP)-26 and tissue inhibitor of MMPs (TIMP)-3 and –4 in normal and ovarian cancer. Gynecol Oncol (submitted)

139. Rhoton-Vlasak A, **Chegini N**, Hardt N Williams RS. (2004) Endometrial Histological Assessment of Norplant Users with Different Uterine Bleeding Pattern Correlates with Elevated Production of IL-13 and IL-15. Fertil Steril (Submitted)

140. Ding L, Xu J, Luo X and **Chegini N** (2004). Gonadotropin releasing hormone analogue (GnRHa) and transforming growth factor beta signal through MAP Kinase pathway to alter the expression of fibronectin, collagen and PAI-1 in leiomyoma and myometrial smooth muscle cells. J Clin Endocrinol Metabol. (In press)

141. Luo X, Ding L and **Chegini N** (2004) Gonadotropin releasing hormone and transforming growth factor beta activate MAP Kinase and differentially regulate fibronectin expression in endometrial epithelial and stromal cells. Am J Physiol (submitted).

INVITED MANUSCRIPTS

1. Rao CV and **Chegini N** (1983) Nuclear receptors for gonadotropins and prostaglandins. In: Evolution of hormone receptor systems. (Bradshow RA and Gill GN, Eds.) Alan R. Liss, Inc., New York. Vol. 6, PP. 413-423.

2. **Chegini N** and Rao CV (1985) Internalization and uptake of ^{3}H-prostaglandin E_1 and $F_{2\alpha}$ into nuclei, lysosomes and golgi of bovine corpora lutea. In: Proceedings of the 5th Ovarian Workshop. (Toft, D.O., and R.J. Ryan eds.) Ovarian Workshop, Champagne, Illinois, Vol. 5, pp. 51-57.

3. Rao CV and **Chegini N** (1988) Epidermal growth factor in human fetal membranes. In: The physiology and Biochemistry of Human Fetal Membranes. (Mitchell BF ed.) Perinatology press, Ithaca, New York., pp. 181-198.

4. **Chegini N** (1988) Absorbable ligating clips: Their ultrastructural characteristics in vivo and in vitro. In: symposium on gynecologic surgery. (Masterson BJ, ed) J.B. Lipincott, Philadelphia. Clinical Obstet. Gynecol. 31:725-735.

5. Rao CV, **Chegini N**, and Lei ZM (1988) Epidermal growth factor in human placenta. In: Placental protein hormones (M. Mochizuki, R Hussa eds.) Elsevier Publishing Co. pp. 171-182.

6. **Chegini N,** Lei ZM and Rao CV (1989) Light and electron microscope immunocytochemical localization of epidermal growth factor receptors in bovine corpora lutea of pregnancy. In: Processing of the 7th Ovarian Workshop. Growth factors and the ovary. (A Hershfield ed.) Plenum Press. pp. 213-220.

7. Rao CV, **Chegini N** and Lei ZM (1989) Immunocytochemical localization of 5- and 12-lipoxygenase and cyclooxygenase in non-pregnant human uteri. In: New Trends in lipids mediators research (U. Zor, Naor Z, Danon A eds.) S. Karger, Basel Switzerland. Vol. 3, pp 283-286.

8. Schultz GS, **Chegini N,** Grant M, Khaw PT and MacKay S (1992) Effects of growth factors on corneal wound healing. Acta Ophthalmologica. 71-77.

9. **Chegini N** (1996). Oviductal-derived growth factors and cytokines: Implication in preimplantion. Seminar Reprod 14:219-229.

10. **Chegini N** and Williams RS (1997). Macrophages and macrophages derived factors in normal reproductive tissues and endometriosis. In Endometrium and Endometriosis. Diamond M and Osteen K eds. Blackwell Sciences Inc. Publisher. pp 140-145.

11. **Chegini N** (1997). The role of growth factors in peritoneal healing. Transforming growth factorβ (TGF-β). Eur J Surg 163 (Supp 557):17-23.

12. Holmdahl L, Risberg, B, Beck DE, Burns JW, **Chegini N** diZerega GS and Ellis H (1997). Adhesion: Pathogenesis and prevention panel discussion summary. Proceeding of the symposium on Adhesions: Pathogenesis and Prevention. Gothenburg, Sweden January, 1996. Eur J Surg. 166 (Supp 577).

13. **Chegini N** (1997). Postsurgical peritoneal repair: Implication of TGF-β. In Pelvic Surgery: Adhesion formation and orevention; Proceeding of the 3rd International Congress on Pelvic Surgery and Adhesion Prevention, San Diego, California February 1996. G. deZeriga et al ed. Springer Verlag Publisher, New York, New York. PP 50-61.

14. **Chegini N** and Williams (2000). Cytokine networks in human endometrium from menstruation to implantation. In; Cytokines in human Reproduction. J. Hill ed. Wiley & Sons Publisher, pp 93-132.

15. **Chegini N** (2000). Implication of growth factors and cytokines in Leiomyomas. In Cytokines and Reproduction. J. Hill ed. Wiley & Sons Publisher. pp 133-162.

16. **Chegini N** (2002). Peritoneal molecular environment, adhesion formation and clinical implication. Front Biosci. 7:e91-115.

17. **Chegini N** (2003). Peritoneal adhesion. Clinical consequences, molecular environment and preventative measurers. Lancet.

18. Chin GA, Schultz GS, **Chegini N** and Diegelmann RF (2004) Biochemistry of wound healing in wound care practice. In; Wound care practice, Sheffield PJ, Fife CE and Smith APS. Edts. Best publishing co. pp49-71.

CONTRIBUTED PAPERS

1. Maclean N, **Chegini N,** Gregory SP and Hilder VA (1978) Transcriptional reactivation of isolated nuclei by cytoplasm. The 37th Annual Meeting of the British Society for Developmental Biology. Oxford, England. Abst. #14.

2. Maclean N, Gregory SP, Hilder VA and **Chegini N**. (1978) The state of nucleus during the differentiation of Xenopus erythroid cell. The 13th international embryological conference, Berlin. Abst. #97.

3. Rao Ch V, Carman FR, Jr., Mitra SB, **Chegini N**. Cook CL and Schultz GS (1982) Topography of human placental receptors for epidermal growth factor (EGF). Endocrinology, Abst. #264.

4. **Chegini N.** and Rao Ch V (1982) Internalization of ^{125}I-human chorionic gonadotropin and ^{3}H-prostaglandins E and PGF_{α} in bovine luteal slices: An electron microscope autoradiographic study. J. Cell Biol. 95, Abst. #9079.

5. Safa AR, **Chegini N** and Tseng MT (1982) Metabolic and morphological alteration in cultured breast tumor cells exposed to mitoxantrone. Am. Assoc. Cancer Res. 24, Abst. #252.

6. Ramani N, **Chegini N**, Rao Ch V and Schultz GS (1983) The presence of epidermal growth factor binding sites in the intracellular organelles of human placenta. Fed. Proc. 42, Abst #862.

7. Rao ChV, Carman FR, Jr., **Chegini N** and G.S. Schultz (1983) Epidermal growth factor binding to human fetal membranes. Endocrinology, 112, Abst. #792.

8. **Chegini N**, N Ramani, R. Vijayakumar and Ch.V. Rao (1983) Morphological and biochemical characterization of small and large bovine luteal cells. J. Cell Biol. 97, Abst. #148.

9. Safa AR, Tseng MT and **Chegini N** (1983) Inhibition of protein synthesis and RNA polymerases by mitoxantrone in cultured breast cancer cells. J Cell Biol. 97, Abst. #460.

10. **Chegini N** and CV Rao (1984) Light and electron microscope autoradiographic study on ^{125}I-epidermal growth factor interaction with term human placenta, fetal membranes and decidua. Society for Gynecologic Investigation. Abst. #235.

11. **Chegini N** and Ch V Rao (1984) Biochemical and quantitative electron microscope autoradiographic (EM-AR) studies on ^{125}I-human chorionic gonadotropin (^{125}I-hCG) internalization in bovine luteal slices. In: The proceedings of the international congress of Endocrinology Abst. #438.

12. **Chegini, N.** and Ch.V. Rao (1984) A quantitative electron microscope autoradiographic study on ^{3}H-PGE$_1$ and ^{3}H-PGF$_{2\alpha}$ internalization in bovine luteal slices. In: The 5th Ovarian Workshop. Abst. #3.

13. **Chegini, N.** and Ch.V. Rao (1984) The appearance of nuclear bodies in luteal slices incubated with human chorionic gonadotropin (hCG). J. Cell Biol. 99, Abst. #794.

14. **Chegini, N.** and Ch.V. Rao (1985) 3 ß-hydroxy steroid dehydrogenase (3 ß-HSD) activity in human placenta, fetal membranes and decidua from mid and full term pregnancy. Society for Gynecologic Investigation. Abst. #382.

15. **Chegini, N.** and Ch.V. Rao (1985) Light and electron microscope autoradiographic studies on ^{125}I-epidermal growth factor (^{125}I-EGF) binding to human placenta, fetal membranes and decidua from mid and term pregnancy. Society for Gynecologic Investigation. Abst. #373.

16. Rao, Ch.V. and **N. Chegini,** N. Wakim and J. Sanfilippo (1985) Prostaglandin (PGE) receptor on different cell types of human uterus during proliferative and secretory phases. Endocrinology. Abst. #914.

17. Rao, Ch.V., Ramani N., **N. Chegini,** P. Woost and G.S. Schultz (1985) Intracellular epidermal growth factor (EGF) binding sites in term placenta. 13th International Congress of Biochemistry. Abst. #TH-571.

18. **Chegini, N.**, Ch.V. Rao, N. Wakim and J. Sanfilippo (1985) Binding sites for epidermal growth factor on different cell types of human uterus during proliferative secretory phases. J. Cell Biol. 101, Abst. #1118.

19. **Chegini, N.**, Ch.V. Rao and J. Sanfilippo (1986) Binding sites for prostacyclin in human and bovine uterus. In: 6th International Conference on prostaglandin and related compounds. Abst. #141.

20. Rao, Ch.V., **N. Chegini** and J. Sanfilippo (1986) Light microscope autoradiographic studies on ^{3}H-Leukotriene C$_4$ binding to human bovine uterus and bovine corpus luteum. In: 6th International conference on prostaglandin and related compounds. Abst. #142.

21. **Chegini, N.** and Ch.V. Rao (1986) Dynamic of nuclear associated granules in bovine luteal cells after treatment with prostaglandins, gonadotropins and cyclic AMP. Biol. Reprod. 34:

Suppl. #1, Abst. #315.

22. **Chegini, N.** and Ch.V. Rao (1986) Influence of gonadotropins, prostaglandins and cyclic AMP on nuclear bodies in bovine luteal cells. In: The 6th Ovarian Workshop, Abst. #6.

23. Rao, Ch.V. and **N. Chegini** (1986) Epidermal growth factor receptors and human placenta. In: 2nd joint meeting of the European and Rochester placental groups. Rolduc The Netherlands. Abst. #1.

24. **Chegini, N.**, J.A. von Fraunhofer and B.J. Masterson (1986) Absorbable ligating clips. American Electron Microscope Society, Ohio Valley Meeting.

25. **Chegini, N.** and Ch.V. Rao (1986) Nuclear volume and chromatin pattern changes in bovine luteal cells following treatment with human chorionic gonadotropin, prostaglandins and cyclic AMP. J. Cell Biol. 104, Abst. #606.

26. **Chegini, N.** and Ch.V. Rao (1987) Prostacyclin binding sites in human uterus. Society for Study of Reprod. Biology of Reproduction (Suppl) 36, Abst. #190.

27. Rao, Ch.V., and **N. Chegini** (1987) Binding sites for Leukotriene C_4 in bovine corpora lutea. Society for Study of Reprod. Biology of Reproduction (Suppl) 36, Abst. #273.

28. **Chegini, N.** and Ch.V. Rao (1987) Leukotriene C_4 binding sites in non-pregnant human uterus. J. Cell Biol. 105, Abst. #1338.

29. Rao, Ch.V. and **N. Chegini** (1988) Prostacyclin (PGI_2) binding to bovine corpora lutea (BCL) of pregnancy: selective enrichment of binding sites in large luteal cells. Taipei Conference on prostaglandin and Leukotriene Research (Taipei, Taiwan). Abst. #S-18.

30. **Chegini, N.** and Ch.V. Rao (1988) Increase of nuclear volume of bovine small (SLC) and large (LLC) luteal cells following treatment in vitro with human chorionic gonadotropin (hCG) and prostaglandin (PG)E_1. 8th International Congress of Endocrinology (Kyoto, Japan), Abst. #16-21-208.

31. Rao, Ch.V. and **N. Chegini** (1988) Leukotriene C_4(LTC_4) and prostacyclin (PGI_2) binding sites in non-pregnant bovine uterine tissue. 8th International Congress of Endocrinology (Kyoto, Japan), Abst. #25-21-001.

32. **Chegini, N.**, Z.M. Lei, Ch.V. Rao and E. Stoelk (1988) Cellular composition of human corpora lutea of various reproductive states.Society for Study of Reprod. Biol. of Reprod. (Suppl) 38, Abst. #400.

33. Stoelk, E., **N. Chegini**, Z.M. Lei, Ch.V. Rao, G. Bryant-Greenwood and J. Sanfilippo (1988). Light and electron microscope immunocytochemical localization of relaxin in human corpora lutea of various reproduction states. Biol. Reprod. (Suppl) vol. 38, Abst. #432.

34. Lei ZM., **N. Chegini**, Ch.V. Rao, P. Bischof and E. Stoelk (1988) Immunocytochemical localization of pregnancy-associated plasma protein-A in human corpora lutea of various reproductive states. 8th International Congress of Histochemistry and Cytochemistry, Washington, D.C., Abst. #381.

35. Lei, Z.M., **N. Chegini** and Ch.V. Rao (1988) Light microscopic immunocytochemical localization of Eicosanoid synthesizing enzymes in bovine corpora lutea. 8th International Congress of Histochemistry and Cytochemistry, Washington, D.C., Abst. #382.

36. **Chegini, N.**, Z.M. Lei, and Ch.V. Rao (1988) Light and electron microscope immunocytochemical localization of epidermal growth factor (EGF) receptors in bovine corpora lutea of pregnancy. 7th Ovarian Workshop, Seattle, Washington, Abst. #15.

37. Metz, S.A., **N. Chegini** and B.J. Masterson (1988) In vivo tissue reactivity and degradation of suture materials. Am. College of Gyn. Abst. #72.

38. **Chegini, N., S.A.** Metz and B.J. Masterson (1988) Tissue reactivity and degradation patterns of absorbable vascular-ligating clips implanted in peritoneum and rectus fascia. Am. College of GYN, Abst. #74.

39. Metz, S.A., **N. Chegini,** J.A. von Fraunhofer and B.J. Masterson (1988) *In vivo* and *in vitro* degradation of monofilament absorbable sutures. Am. College of GYN, Abst. #73.

40. Rao, Ch.V., Z.M. Lei, and **N. Chegini** (1988) Cellular distribution of 5-lipoxygenase, 12-lipoxygenase and cyclooxygenase in non-pregnant human uteri. 2nd International Conference on Leukotrienes and prostanoids in health and disease. Jerusalem, Israel., Abst. #S49.

41. Bailey-Pridham, D., Z.M. Lei, **N. Chegini**, Ch.V. Rao and M. Yussman (1988) The presence of 5- and 12-lipoxygenase and cyclooxygenase enzymes in human granulosa cells from preovulatory follicles. 2nd International Conference on Leukotrienes and prostanoids in health and disease. Jerusalem, Israel., Abst. #P18.

42. Lei, Z.M., **N. Chegini**, Ch.V. Rao, M.W. Alila and W. Hansel (1989) Epidermal growth factor receptors (EGF-R) in bovine corpora lutea during the cycle. J. Cell Biol. 107, Abst. #3940.

43. Rao, Ch.V., **N. Chegini** and Z.M. Lei (1989) Nuclei and cytoplasmic organelles of human placental, fetal membrane and decidual cells from mid and term pregnancy contain epidermal growth factor (EGF) receptors. J. Cell Biol. 107, Abst. #3941.

44. **Chegini, N.**, A.M. Lei, Ch.V. Rao, H.W. Alila and W. Hansel (1989) Cellular distribution of cyclooxygenase (CO), prostacyclin (PGI_2-S) and thromboxane A_2(TXA_2-S) synthase in bovine corpora lutea (bCL) during the cycle. J. Cell Biol. 107, Abst. #4885.

45. Goldin, A.G., **N. Chegini** and A.R. Safa (1989) The inhibitory effect of digoxin on MCF-7

and MDA-MB-231 human breast tumor cell lines. J. Cell Biol. 107, Abst. #226.

46. Reshef, E., Z.M. Lei, **N. Chegini,** Ch.V. Rao and J.L. Lubosky (1989) The presence of human chorionic gonadotropin/luteinizing hormone (hCG/LH) receptors in non-pregnant human uteri. Society of Gynecologic Investigation. Abst. #585.

47. Bailey-Pridham, D., Z.M. Lei, **N. Chegini,** Ch.V. Rao and M.A. Yussman (1989) Immunocytochemical localization of 5- and 12-lipoxygenase and cyclooxygenase in human granulosa cells from preovulatory follicles. Society for Gynecologic Investigation. Abst. #380.

48. Rossi, M.J., **N. Chegini** and B.J. Masterson (1990) Human myometrial smooth muscle cells response to growth factors. Symposium on Growth Factors in Reproduction. Serono Symposia USA. Abst. #34.

49. Rossi, M.J., **N. Chegini** and B.J. Masterson (1990) Regulation of human endometrial cell proliferation by growth factors. Biol. Reprod. Abst. #97.

50. Williams, R.S. and **N. Chegini** (1990) Localization of TGF-α and TGF-ß in human uterine and ovarian tissue. The American Fertility Society Annual Meeting. Abst. # S 5-13.

51. **Chegini, N.**, M.J. Rossi, G.S. Schultz, W.A. Dunn, Jr. and B.J. Masterson (1991) Cellular distribution of EGF receptor in fascia and peritoneum during healing. An autoradiographic and immunocytochemical study. Wound Healing Society First Annual Meeting. Galveston, Texas.

52. **Chegini, N**. and B.J. Masterson (1991)Immunocytochemical localization of EGF, TGF-α and TGF-ß in fascial and peritoneal injury during healing. Wound Healing Society First Annual Meeting. Galveston, Texas.

53. **Chegini, N.**, M.J. Rossi and B.J. Masterson (1991) Presence of 5-lipoxygenase and specific leukotrienes (LTC_4, LTD_4 but not LTB_4) binding sites in fascial and peritoneal injury during healing. Wound Healing Society First Annual Meeting. Galveston, Texas.

54. **Chegini, N**. and B.J. Masterson (1991) Cellular localization of cyclooxygenase and thromboxane A_2 synthase during the wound healing process in fascial and peritoneal injury. Wound Healing Society First Annual Meeting. Galveston, Texas.

55. **Chegini, N**. and K.C. Flanders (1991) Transforming growth factor-β (TGF-β) Type I and Type 2 localization and their selective compartmentation in human ovarian tissue of various reproductive states. Society for Gynecologic Investigation. Abst. #507.

56. Williams, RS, AMK. Rossi, **N. Chegini** and G.S. Schultz (1991) Effect of transforming growth factor beta on postoperative adhesion formation and intact peritoneum. Society for Gynecologic Investigation. Abst. #466.

57. **Chegini, N.**, JS. Simms, R.S. Williams and A.M.K. Rossi (1991) Identification of transforming growth factors alpha and beta (TGF-α and TGF-β) in postoperative adhesion formation. Society for Gynecologic Investigation. Abst. #465.

58. Rossi, M.J., **N. Chegini** and B.J. Masterson (1991) Insulin-like growth factor I Stimulation of human endometrial and myometrial cells mitogenesis in vitro. Society for Gynecologic Investigation. Abst. #240.

59. Simms, J.S., **N. Chegini**, R.S. Williams, A.M.K. Rossi and W.A.Dunn, Jr. (1991) Identification of epidermal growth factor, transforming growth factor-alpha, and epidermal growth factor receptor in surgically-induced endometriosis in rat. The American College of Obstetric Gynecology Annual Meeting. Abst. #120.

60. Rossi, M.J., W. Pawlina, L.H. Larkin and **N. Chegini** (1991) Presence of relaxin binding sites in human myometrial smooth muscle cells and its inhibitory effect on EGF action in culture. Biol. Reprod. Suppl. (37) Abst. #57.

61. Miller, G.A., **N. Chegini,** W.C. Buhi, F.W. McLean, L.B. Landy, I.K. Stone, K.S. Williams and B.J. Masterson (1991) Immunolocalization of retinol-binding protein in human uterine tissue. Am. Fertil. Society. Annual Meeting Abst. # P 003-S85.

62. Bass, B.E., R.S. Williams, A.M. Rossi and **N. Chegini** (1991) Epidermal growth factor(EGF) infusion in rat uterus horns stimulate as estrogenic effect. Am. Fertil. Society. Annual Meeting Abst. # O 085-S36.

63. **Chegini, N.**, M.J. Rossi and B.J. Masterson (1991) Production of EGF, PDGF-AB, and presence of EGF, PDGF-BB and IGF-I receptors and their action in human uterine tissue. J. Cell Biol. 115: Abst. #69.

64. Rossi, M.J., **N. Chegini,** K.C. Flanders and G.A. Ksander (1992) Tissue localization and in vitro action of transforming growth factor βs in human uteri. 39th Annual Meeting of Society for Gynecologic Invest. Abst. #578.

65. **Chegini N.**, KC. Flanders, FW. McLean and BJ. Masterson (1992) Localization of EGF, TGF-α, TGF-β1, TGF-β2 and EGF receptor in human fallopian tubes of various reproductive states. 39th Annual Meeting of Society for Gynecologic Invest. Abst. #586.

66. Rossi, M.J., X-M. Tang, B.J. Masterson and **N. Chegini** (1992) The direct proliferative action of macrophages in human endometrial and myometrial tissue. 39th Annual Meeting of Society for Gynecologic Invest. Abst. #577.

67. **Chegini, N.**, W.E. Chafe, W.A. Dunn and B.J. Masterson (1992) Presence of epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and epidermal growth factor receptor in human ovarian carcinomas. Gynecol. Onc. 45(1) Abst# 84, Society Gynecologist Oncology Annual Meeting.

68. **Chegini, N.**, W.E. Chafe and K.C. Flanders (1992) Immunolocalization of transforming growth factor β1 and β2 in human ovarian tumors. Gynecol. Onc. 45(1) Abst# 82, Society Gynecologist Oncology Annual Meeting.

69. Schultz GS, PT Khaw, SLD MacKay, **N Chegini**, DS Rotatori, J Adams and RW Shimizu (1992). Human corneal epithelium: Autocrine production of transforming growth factor alpha mRNA and protein. J. Invest. Opthalmol. Vis. Sci. 33 (Suppl), Abst #1686.

70. Tang, X-M., **N. Chegini**, M.J. Rossi, M.F. Fay and B.J. Masterson (1992) The effect of surgical glove powder on human skin fibroblasts and monocyte/macrophages proliferation. European Tissue Repair Society. Malmo Sweden Abst #82.

71. Tang, X-M., **N. Chegini,** M.J. Rossi, M.F. Fay and B.J. Masterson (1992) The direct action of surgical glove powder on fibroblast and monocyte/macrophage proliferation in co-culture. European Tissue Repair Society. Melmo Sweden Abst #83.

72. **Chegini, N.**, W.E. Chafe, W.A. Dunn and B.J. Masterson (1992) Presence of epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and epidermal growth factor receptor in human ovarian carcinomas. Ninth International Congress of Endocrinology. Nice, France. Abst# 1855.

73. **Chegini, N.**, W.E. Chafe and K.C. Flanders (1992) Immunolocalization of transforming growth factor β1 and β2 in human ovarian tumors. Ninth International Congress of Endocrinology. Nice, France Abst #1854.

74. Connor, E.E., **N. Chegini**, and K.T. Shiverick (1993) The effect of Benzo(a)Pyrene on epidermal growth factor receptor, cell proliferation and ^{3}H-thymidine incorporation in BeWo and Jeg-3 human placental cytotrophoblast cell lines. Annual Endocrine Society Meeting. Abst# 1193.

75. Tang, X-M., **N. Chegini,** M.F. Fay and B.J. Masterson (1993) The direct action of surgical glove powder on monocyte/macrophages and lymphocyte cytokine production *in vitro*. European Society for Surgical Research. Turko Finland Abst #152.

76. Tang, X-M., **N. Chegini,** M.F. Fay and B.J. Masterson (1993) The effect of surgical glove powder on monocyte/macrophage and lymphocyte eicosanoids production *in vitro*. European Society for Surgical Research. Turko Finland Abst #155.

77. Pfeifer, T.L. and **N. Chegini** (1993) Immunohistochemical localization of insulin-like growth factor(IGF-I), IGF-I receptor (IGF-IR) and IGF binding proteins 1-4 in human fallopian tube at various reproductive stages. 49th Annual Meeting of the American Fertility Society. Montreal Canada. Abst #P-0121.

78. Pfeifer, T.L., S. Juneja, X-M. Tang, R.S. Williams and **N. Chegini** (1993) Macrophage

enhancement and inhibition of embryo development. 49th Annual Meeting of the American Fertility Society. Montreal Canada. Abst #O-0007.

79. **Chegini, N.**, X-M. Tang, M.J. Rossi, S. Barnes, C.J.M. Kane and B.J. Masterson (1993) Detection of Relaxin in rat and human Skin wound and its effect in skin fibroblast i*n vitro*. Joint meeting of the European tissue repair society and the Wound healing society. Amsterdam. Abst #33.

80. Juneja, S., S. Kippersztok, S.J. Darkin-Rattray and **N. Chegini** (1993) Benzo(a)pyrine adversely effects ovulation in mice. 49th Annual Meeting of The American Fertility Society. Montreal Canada. Abst #0-0156.

81. Juneja, S., R.S. Williams, **N. Chegini** and G. Ksander (1993) Ovarian intrabursal administration of recombinant TGF-β1 inhabits follicle rupture in gonadotropin-primed mouse. 49th Annual Meeting of the American Fertility Society. Montreal Canada. Abst #0-0152.

82. Stone, I.K. and **N. Chegini** (1993) Identification of epidermal growth factor, transforming growth factor-alpha and epidermal growth factor receptor in Vulvar Lichen Sclerosis. American Vulvar Society Meeting.

83. **Chegini, N.** and Y. Zhao (1994) Human fallopian tubes express granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors and contain the immunoreactive gene product for GM-CSF. Society for Gynecologic Investigation 41st Annual Meeting. Abst# P259.

84. Tang, X-M., Y. Zhao and **N. Chegini** (1994) Human fallopian tube expresses mRNA and protein for relaxin and contain specific relaxin receptor. Society for Gynecologic Investigation 41st Annual Meeting. Abst.#O210.

86. Zhao, Y., and **N. Chegini** (1994) The expression and cellular localization of TGF-β1, TGF-β2, TGF-β3 and TGF-β Type II receptor mRNA and protein and ^{125}I-TGF-β1 binding sites in human fallopian tube. Society for Gynecologic Investigation 41st Annual Meeting. Abst# O209.

87. Buhi, W., **N. Chegini**, H. Rong and I. Alvarez (1994) Immunolocalization of transforming growth factor (TGF-β) TGF-β1, TGF-β2, and TGF-β type II receptor protein in the porcine oviduct. Society for Gynecologic Investigation 41st Annual Meeting. Abst.# P256.

88. Pfeifer, T.L., S.C. Juneja, R.S. Williams and **N. Chegini** (1994) The effect of macrophages on trophoblast outgrowth *in vitro*. Society for Gynecologic Investigation 41st Annual Meeting. Abst #P312.

89. **Chegini, N.**, H. Rong, Y. Zhao and R.S. Williams. (1994) Gonadotropin hormone releasing hormone agonist (GnRHa) suppresses the expression of mRNA and proteins for growth factors and their receptors in uterine leiomyomata. Society for Gynecologic Investigation 41st Annual

Meeting. Abst #P260.

90. Juneja, S.C., T. Pfeifer, R.S. Williams and **Chegini N** (1994) Neem oil inhibits mouse preimplantation embryos and implantation *in vitro*. Society for Gynecologic Investigation 41st Annual Meeting. Abst #P243

91. Faber BM, SA Metz, and **Chegini N.** (1994) Immunolocalization of growth factors and eicosanoid pathway enzymes in myometrium, placenta, and fetal membranes from normal versus failed labor. Society for Gynecologic Investigation 41st Annual Meeting. Abst #P407.

92. Zhao Y, Tang X-M, and **Chegini N.** (1994) The expression of granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors mRNA and immunoreactive gene product for GM-CSF in human uterine tissue. Society for Gynecologic Investigation 41st Annual Meeting. Abst #O117.

93. Tang X-M, Zhao Y, Abu-Rustum RS, Masterson BJ and **Chegini N.**(1994) Human uterus expresses TGF-β1, TGF-β2, TGF-β3 and TGF-β Type II receptor mRNA and proteins and contains ^{125}I-TGF-β1 binding sites. Society for Gynecologic Investigation 41st Annual Meeting. Abst. #O202.

94. Zhang L, Connor EE, **Chegini N** and Shiverick KT (1994) The effect of Benzo(a)Pyrene on epidermal growth factor receptor protein and human chorionic gonadotropin secretion by BeWo and Jeg-3 human placental trophoplasts cell lines. Annual Meeting of Society of Toxicology.

95. Schultz GS, Zhao Y, Tarnuzzer RW and **Chegini N** (1994) Detection of GM-CSF mRNA and protein in human corneal epithelial cells; possible corneal defense from infection. J. Invest. Opthalmol. Vis. Sci. (Suppl), Abst # 3168.

96. Juneja SC, Pfeifer TL, Tang X-M, Williams RS and **Chegini N** (1994) Inhibition of in vitro fertilization of mouse ova by conditioned medium from activated macrophages. Society for the Study of Reprod Annual meeting Ann Arbor Michigan Abst #333

97. Zhao Y, and **Chegini N** (1994) The expression of granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors mRNA and immunoreactive gene product for GM-CSF in human ovarian tissue. 10th Ovarian Workshop, Ann Arbor Michigan. Abst. #.

98. Rong H, Tang X-M, Zhao Y, Juneja SC, **Chegini N**, Beezhold D, Fay M and Masterson BJ (1994) Intraperitoneal environment and adhesion formation: the role of glove powders and latex proteins. European Tissue Repair Society Oxford England Abst. #87.

99. Humphreys-Beher MG, Purushotham K, Wheeler T, Stewart C, **Chegini N**, and Schultz GS (1994). Salivary gland synthesis and saliva levels of TGF-α. J Dental Res 73:310 Abst. #1664.

100. Juneja SC, Williams RS and **Chegini N** (1994) Effect of Andordrin on the development of

mouse preimplantation embryos in vitro. Fiftieth Annual meeting of the American Fertility Society, San Antonio TX 1994. Abst #O-176.

101. Rong H, Williams RS, and **Chegini N.** (1995) The Direct action of gonadotropin releasing hormone agonist and antagonists on human myometrial smooth muscle cell growth and TGF-β1 expression in vitro. J Soc Gynecol Invest 2:Abst.#O57.

102. Zhao Y, Rong H, and **N. Chegini**. (1995) Expression and selective localization of granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF α and β receptors mRNA and protein in human ovary. J Soc Gynecol Invest 2:Abst. #P403.

103. Tang X-M, Zhao Y, Dou Q, Tarnuzzer RW, Schultz GS and **Chegini N.** (1995) Regulation of TGF-β1 mRNA and protein expression by granulocyte-macrophage colony stimulating factor (GM-CSF) in human endometrium. J Soc Gynecol Invest 2: Abst. #P399

104. Zhao Y, Tarnuzzer RW, Williams RS, Schultz GS and **Chegini N.** (1995) Gonadotropin releasing hormone agonist (GnRHa) suppresses the expression of TGF-βs and TGF-β type II receptor mRNA in uterine leiomyomata. J Soc Gynecol Invest 2: Abst.#P405

105. Zhao Y, Tarnuzzer RW, Metz SA, Schultz GS and **Chegini N.** (1995) Estrogen replacement therapy in postmenopausal women enhances the expression of TGF-β mRNA in the vaginal epithelial tissue. J Soc Gynecol Invest 2; Abst#.

106. Tang X-M, Ghahary A and **Chegini N** (1996). The interaction between transforming growth factor β and relaxin leads to modulation of matrix and matrix metalloproteinases expression in human uterus. J Soc Gynecol Invest. 3; Abst#

107. Tarnuzzer RW, Schultz SG, and **Chegini N** (1996). Differential expression of matrix metalloproteinases and their tissue inhibitors in leiomyomata from women treated with gonadotropin releasing hormone agonist. J Soc Gynecol Invest. Abst#206

108. Dou Q, Tang X-M, Kipersztock S, and **Chegini N.** (1996) The interactive relationship between transforming growth factor β and granulocyte-macrophage stimulating factor in human uterus. J Soc Gynecol Invest. J Gynecol Invest. 3: Abst# 261.

109. Rosa C, Dou Q and **Chegini N** (1996). The expression of transforming growth factor β1 (TGF-β1) and TGF-β type I and type II receptors are differentially regulated in human myometrium from chorioamnionitis compared to failed labor-induction and no laboring patients. J Soc Gynecol Invest. 3: Abst# 296.

110. Dou Q, Tang X-M, Williams RS, Gelder M, and **Chegini N** (1996). Antiprogestins differentially regulate the expression of TGF-β1 and GM-CSF in normal and carcinoma cells of human endometrium. J Soc Gynecol Invest. 3: Abst# 259.

111. Habibi H, Dou Q and **Chegini N.** (1996) Gonadotropin Releasing hormone (GnRH) effect on

production of TGF-β1 and TGF-β2 receptor in the human hepatoma carcinoma cell line HepG2. Interml Endocrine Sco Meeting. San Francisco CA. Abst #P3-710

112. Chegini N, Schultz G, Lynch S, and Dunn R (1996). Immunohistochemical evaluation of MMPs, growth factors and receptors in sequential biopsies of chronic human pressure ulcers. Wound Res Reg 4: Abst#A136.

113. Dou Q, Williams RS, and **Chegini N.** (1997) Inhibition of macrophage-derived TGF-β1 expression by antisense oligonucleotide alters their growth, aggregation and integrins mRNA expression. J Soc Gynecol Invest. 4:Abst# 16

114. Dou Q, Williams RS, and **Chegini N.** (1997) Evidence that human endometrium throughout the menstrual cycle expresses mRNA for integrins. A competitive quantitative RT-PCR using a multiprimer external synthetic RNA standard. J Soc Gynecol Invest. 4:Abst#215

115. Kipersztok, S. Juneja, S and **Chegini N** (1997). Evidence that Benzo(a)pyrene induces disruption of follicular development and ovulation. J Soc Gynecol Invest. 4:Abst#411

116. Faber B, Coddington CC, **Chegini N** and Mahony M (1997). Edidence for impaired sperm-zona pellucida binding in endometriosis associated infertility. J Soc Gynecol Invest. 4: Abst# 598.

117. Tarnuzzer RW, **Chegini, N**, Hutcheson KA, and Lambert SR (1997) Immunolocalization of TGF-β1, TGF-β type I and IIRc, IGF-I and IGF-I Rc in juvenile and adult monkey eyes. J. Invest. Opthalmol. Vis. Sci. (Suppl), Abst #3518.

118. Drack AV, **Chegini N**, Fernandez A, Capone A, Hutcheson KA, and Tarnuzzer RW (1997) Association of TGF-β with decreased axial elongation following diode laser photocoagulation in infant monkey eyes. J. Invest. Opthalmol. Vis. Sci. (Suppl), Abst # 3519.

119. deMoraes AAS, Paula-Lopes FF, **Chegini N** and Hansen PJ (1997). Immunohistochemical localization of granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor-β1 (TGF-β1) in the reproductive tract of cyclic cows. J Amim Sci 75 (Suppl 1) 141.

120. Flak P, Holmdahl L, Iversson ML and **Chegini N.** (1997). Transforming growth factor beta, a paracrine regulator of mesothelial cell fibrinolysis. International Congress of Peritoneal Tissue Repair. The 4th Peritoneum and Peritoneal Access Meeting, Gotheborg Sweden. Abst #13.

121. Bergstrom M, Dou Q, Kostseos K, Iversson ML, Falk P, **Chegini N** and Holmdahl L (1997). Overproduction of TGF-β is associated with adhesion formation and fibrinolytic impairment. International Congress of Peritoneal Tissue Repair. The 4th Peritoneum and Peritoneal Access Meeting, Gothenborg Sweden. Abst #14.

122. Holmdahl L, Blum R, **Chegini N**, Iversson ML, Bergstrom M (1997). Coagulation and

fibrinolysis in peritoneal tissue. International Congress of Peritoneal Tissue Repair. The 4th Peritoneum and Peritoneal Access Meeting, Gothnborg Sweden. Abst #27.

123. Holmdahl L, Tang XM and **Chegini N** (1997). Contamination of surgical gloves: Implication on peritoneal tissue repair. International Congress of Peritoneal Tissue Repair. The 4th Peritoneum and Peritoneal Access Meeting, Gothnborg Sweden. Abst #38.

124. **Chegini N**, Rong H, Bennett B and Stone IK (1998). Molecular analysis of intraperitoneal environment and its relashionship to adhesion formation and endometriosis. Society for Gynecologic Investigation 45th Annual Meeting. Abst. #299

125. Dou Q and **Chegini N** (1998). An inverse relashionship between TNF-α and TNF-α receptor mRNA expression in human endometrium. Society for Gynecologic Investigation 45th Annual Meeting. Abst. #283

126. Dou Q, Ma C, Williams RS, and **Chegini N** (1998). The identification of several differentially expressed noval genes in leiomyoma and myometrium. Society for Gynecologic Investigation 45th Annual Meeting. Abst. #581

127. Ma C, Duo Q and **Chegini N** (1998). Modulation of interleukin (IL), IL-2, IL-5, IL-6, IL-12, IL-13 and IL-16 expression in human mesothelial cells, macrophage and lymphocyte exposed to glove powders, latex proteins and LPS. Am Society for Reprod Med. Abst. # P892

128. Ma C, Tarnuzzer RW and **Chegini N** (1998) Modulation of matrix metalloproteinases and tissue inhibitor of MMPs (TIMPs) expression in human myometrial smooth muscle cell, mesothelial cell and macrophage by transforming growth factor β1. Am Society for Reprod Med. Abst.#O134.

129. **Chegini N**, Zhao Y, Kotseos K, Bennett B, McLean F, Diamond MP, Holmdahl L, Nickerson C, Burns J and The Peritoneal Healing and Adhesion Multi University Study (PHAMUS) group (1998). Comparative analysis of matrix metalloproteinase (MMP-1), tissue inhibitor of MMPs (TIMP-1) and MMP-1/TIMP-1 complex expression in intraperitoneal environment and their relation to adhesion development. Am Society for Reprod Med. Abst.O69.

130. **Chegini N**, Zhao Y, Kotseos K, Bennett B, McLean F, Diamond MP, Holmdahl L, Nickerson C, Burns J and The Peritoneal Healing and Adhesion Multi University Study (PHAMUS) group (1998). Matrix metalloproteinase (MMP-1), tissue inhibitor of MMPs (TIMP-1) and MMP-1/TIMP-1 complex expression in intraperitoneal environment. International Soc laparoscopy.

131. Holmdahl L, Blum R, Iversson ML, Bergstrom M and **Chegini N** (1998). Coagulation and fibrinolysis in peritoneal tissue. Eight Annual Meeting of The European Tissue Repair Society. Wound Rep. & Regen 6; A487.

132. Holmdahl L, Falk P, Iversson ML, Palmgren I, Hedggren M, **Chegini N** and Diamond MP,

(1998). Plasmin activator and inhibitor in abdominal wall and peritoneal cavity. Eight Annual Meeting of The European Tissue Repair Society. Wound Rep. & Regen 6; A487.

133. Holmdahl L, Bergstrom M, Dou Q, Kostseos K, Iversson ML, Falk P and **Chegini N.** (1998). Overexpression of TGF-β1 is associated with adhesioon formation and fibrinolysis impairment. Eight Annual Meeting of The European Tissue Repair Society. Wound Rep. & Regen 6; A487

134. Ghellai, AM, **Chegini, N**, Stucchi, AF, Ma, C, Kaseta, JM, Andry, CD, Burns, JW, Skinner, KC and Becker, JM (1998). The role of transforming growth factor beta-1 (TGF-β1) in Peritonitis-induced adhesions.Thirty-Ninth Annual Meeting of the Society of Surgery of the Alimentary Tract, New Orleans, LA, May 17-21, 1998 Gastroenterology 114:A1390, 1998.

135. **Chegini N**, Kotseos K, Patel A, Ma C and Grant M (1999).The effect of overexpression of plasminogen activator inhibitor (PAI-1) and aging on intraperitoneal adhesion formation in mice. Society for Gynecologic Investigation 46h Annual Meeting. Abst. #86

136. Ma C, Buie M, Gao Y and **Chegini N** (1999). mRNA expression of interleukines (IL) IL-13, IL-15, and IL-16 in human endometrium and fallopian tubes. Society for Gynecologic Investigation 46h Annual Meeting. Abst. #638.

137. **Chegini N**, Zhao Y, Kotseos K, Bennett B, Diamond MP, Holmdahl L, Skinner and The Peritoneal Healing and Adhesion Multi University Study (PHAMUS) group (1999). The expression of matrix metalloproteinase (MMPs) and tissue inhibitor of MMPs (TIMPs) in various intraperitoneal tissues and their relation to adhesion development. Society for Gynecologic Investigation 46th Annual Meeting. Abst. #738.

138. Diamond MP, Yelian FD, Zhang W, **Chegini N**, Holmdahl L and the PHAMUS group (1999). Vascular endothelial growth factor levels are elevated in adhesion tissues in human. Society for Gynecologic Investigation 46th Annual Meeting. Abst. #739.

139. Svinarich DM, Zaher FM, Gonik B, Holmdahl L, **Chegini N**, Diamond MP and the PHAMUS group (1999). Adhesion development and the expression of endothelial nitric oxide synthesis. Society for Gynecologic Investigation 46th Annual Meeting. Abst. #740.

140. Leach RE, Saed G, Zhang W, Holmdahl L, **Chegini N,** Diamond MP and the PHAMUS group (1999). Human mesothelial cells hypoxia induces hypoxia inducible factor-1α. Society for Gynecologic Investigation 46th Annual Meeting. Abst. #741.

141. Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP and the PHAMUS group (1999). Collagen I and III production by human peritoneal mesothelial cells in response to hypoxia and TGF-β1 treatments. Society for Gynecologic Investigation 46th Annual Meeting. Abst. #746.

142. Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP and the PHAMUS group (1999). Transforming growth factor beta isoforms (TGF-βs) production by human peritoneal

mesothelial cells in response to hypoxia treatments. Society for Gynecologic Investigation 46h Annual Meeting. Abst. #747.

143. **Chegini N**, Zhao Y, Kotseos K, Bennett B, Diamond MP, Holmdahl L, Skinner and The Peritoneal Healing and Adhesion Multi University Study (PHAMUS) group (1999). Expression of matrix metalloproteinase (MMPs) and tissue inhibitor of MMPs (TIMPs) in various intraperitoneal tissues and their relation to adhesion development. Keystone Symposia, Durango CO. Abst. #99.

144. Holmdahl L, Falk P, Iversson ML, Palmgren I, Hedggren M, **Chegini N**, Diamond MP, Skinner K and the PHAMUS group (1999). Plasmin activator and inhibitor in peritoneal environment. International Congress on Peritoneal Repair and Adhesion (PAX) and International mesothelioma Interest Group Combined Meeting. Granthan, England (Abst #6).

145. **Chegini N**, Kotseos K, Zhao Y, Patel A , Bennett B, MP Diamond, L Holmdahl, Nickerson C, K. Skinner and the PHAMUS Group (1999). Matrix metalloproteinase (MMPs) and tissue inhibitor of MMPs (TIMPs) in peritoneal tissues from variou sites. International Congress on Peritoneal Repair and Adhesion (PAX) and International mesothelioma Interest Group Combined Meeting. Granthan, England (abst #12).

146: Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP and the PHAMUS group. Collagen I and III expression in human peritoneal mesothelial cells. International Congress on Peritoneal Repair and Adhesion (PAX) and International mesothelioma Interest Group Combined Meeting. Granthan England, 1999 (Abst #X1A).

147. Saed G, Zhang W, **Chegini N**, Holmdahl L, Diamond MP and the PHAMUS group. Hypoxia alters the production of transforming growth factor beta isoforms (TGF-βs) by human peritoneal mesothelial cells.International Congress on Peritoneal Repair and Adhesion (PAX) and International mesothelioma Interest Group Combined Meeting. Granthan England, 1999 (Abst #X1B).

148. Peck LS, **Chegini N**, Holmdahl L Beltran C, Hammer ML, Kiribuchi K, Roy P, Lye V and Goldberg EP. rTGF-β2 stimulated adhesion formation in a rat cecal model: effect of timing on rTGF-β2 adminstration. International Congress on Peritoneal Repair and Adhesion (PAX) and International mesothelioma Interest Group Combined Meeting. Granthan England, 1999 (Abst #X3).

149. **Chegini N**, K Kotseos, Zhao Y, F. McLean, B. Bennett, MP Diamond, L Holmdahl, J. Burns. TGF-β1 AND TGF-β3 are differentially expressed in serosal surfaces of several human intraperitoneal tissues and peritoneal adhesions. Society for Gynecologic Investigation 47h Annual Meeting, 2000 (Abst #51).

150. **Chegini N,** Kotseos K, Gao Y, Patel A , RS. Williams, MP Diamond, L Holmdahl, and K. Skinner and the PHAMUS Group. Differential expression of intergrin αv and β3 in human intraperitoneal tissues and adhesions. Society for Gynecologic Investigation 47th Annual

Meeting, 2000 (Abst #563).

151. Ma C and **N. Chegini**. The expression of gonadotropin releasing hormone (GnRH) and GnRH receptor and the direct action of GnRH anologes on myometrium and leiomyoma. Society for Gynecologic Investigation 47th Annual Meeting, March 2000 (Abst # 324).

152. M. Mrstik, K Kotseos, C. Ma and **N. Chegini**. Increased expression of interferon-gamma inducible protein-10 (IP-10) mRNA during surgically-induced peritoneal injury. Society for Gynecologic Investigation 47th Annual Meeting, March 2000 (Abst # 828).

153. K Kotseos and **N. Chegini**. The expression of plasminogen activator inhibitor (PAI-1) during the peritoneal wound healing following surgically-induced injury in mice. Society for Gynecologic Investigation 47th Annual Meeting, March 2000 (Abst #561).

154. **N. Chegini**, C Ma and RS Williams. Alteration of transforming growth factor beta expression and autocrine/paracrine actions in leiomyoma smooth muscle cells by gonadotropin releasing hormone analogue (GnRHa), anti-estrogen and anti-progestins. American Society for Reproductive Med. Annual Meeting, October 2000, Abst. # p487.

155. Sui B and **N Chegini**. Alteration of transforming growth factor beta signal transduction in leiomyoma and myometrium by gonadotropin releasing hormone analogue (GnRHa). Society for Gynecologic Investigation 48th Annual Meeting, March 2001.

156. **N Chegini** and L Kornberg. Gonadotropin Releasing Hormone analogues (GnRHa) therapy alters signal transduction pathways that involves ERK and Focal Adhesion Kinases in leiomyoma. The Society for Gynecologic Investigation 48th Annual Meeting, March 2001.

157. **N Chegini**, D Ripley, and BA. Ripps. Expression of Smads, Transforming growth factor beta signal transduction protein, in normal endometrium and endometrial cancer. The Society for Gynecologic Investigation 48th Annual Meeting, March 2001.

158. **Chegini N**, Ma C, Roberts M and Ripps BA. Altered Expression of IL-13 and IL-15 in human endometrium with recurrent spontaneous abortion. Society for Gynecologic Investigation 48th Annual Meeting, March 2001.

159. Xu J, Luo X and **Chegini N**. Alteration of TGF-β Signal Transduction Pathway In Leiomyoma and Myometrial Smooth Muscle Cells By GnRHa. Society for Gynecologic Investigation 48th Annual Meeting, March 2002.

160. Luo X, Xu J, and **Chegini N.** Modulation of TGF-β Signal Transduction Pathway in Human Endometrial and Stromal Cells by GnRHa. Society for Gynecologic Investigation 48th Annual Meeting, March 2002.

161. **Chegini N,** A Rhoton-Vlasak, Q-X Sang a R.S Williams. Increased expression of MMP-26, TIMP-3 and TIMP-4 in endometrium of women with abnormal uterine bleeding. Society for

Gynecologic Investigation 48th Annual Meeting, March 2002.

162. Rhoton-Vlasak. A. Gleich, GJ. Bischof, P and **N Chegini**. Comparative analysis of major basic protein and pregnancy-associated plasma protein–A expression in human ovarian tissue Society for Gynecologic Investigation 48th Annual Meeting, March 2002.

163. Tunuguntla R, Ripley D, Sang Q-X-A and **Chegini N.** The Expression of matrix matelloproteinase (MMP)-26 and tissue inhibitor of MMPs (TIMP)-3 and –4 in benign endometrium and endometrial cancer. J Sco Gynecol Investig. 9; Abst #866. Society for Gynecologic Investigation 48th Annual Meeting, March 2002.

164. **Chegini N,** Varela J, and Williams RS (2002). Gene expression profile of leiomyoma and matched unaffected myometrium and alterations induced by GnRHa therapy. 11th World Congress on Human Reproduction. Montreal, Canada.

165. T. Medrano, **N. Chegini** and K. T. Shiverick. Benzo (A) Pyrene induces a shift to a Th1 cytokine expression profile in human endometrial RL95-2 cells. IFPA meeting, Australia October 2002.

166. T. Medrano, **N. Chegini** and K. T. Shiverick. Benzo(A)pyrene induces a shift to a Th1 cytokine Expression profile in human endometrial RL95-2 cells. IFAS Meeting in Sidney, Australia, October 2002.

167. Xu J and **Chegini N** Activation of MAPK Pathway by Transforming Growth Factor beta and Gonadotropin Releasing Hormone Analogue (GnRHa) in Leiomyoma and Myometrial Smooth Muscle Cells. Society for Gynecological Investigation 2003. Abst # 125.

168. Luo X and **Chegini N.** Transforming Growth Factor beta and Gonadotropin Releasing Hormone Analogue Recruit and Activate MAPK in Endometrial Epithelial and Stromal Cells. Society for Gynecological Investigation 2003. Abst #82.

169. Ripley D, Shoup B, Majewski A, **Chegini N**. Elevated Expression of IL-13 and IL-15 in Human Ovarian Carcinoma. Society for Gynecological Investigation 2003 Abst #449.

170. T. Medrano, N. Chegini and K.T. Shiverick. Th1 cytokine expression is upregulated by Aryl Hhyrocarbon receptor agonist in human endometrial RL95-2 cells. European Placenta Group Meeting, Sept 24-28, 2003, Mainz, Germany.

171. Luo X, Ding L, Xu J, Willimas RS and **Chegini N**. (2004) Gene expression profiling of leiomyoma, myometrium and their isolated smooth muscle cells and regulation by gonadotropin releasing hormone analogue (GnRHa) and TGF-b. Soc for Gynecol Invest Annual Meeting (accepted for presentation)

172. Ding L, Luo X and **Chegini N**. (2004) The expression of IL-13 and IL-15 in leiomyoma and myometrium and their influence on TGF-b and proteases expression in leiomyoma and

myometrial smooth muscle cells and SKLM, leiomyosarcoma cell line. Soc for Gynecol Invest 51 Annual Meeting (accepted for presentation and selected for traveling award)

173. Li R, Luo X, Ding L, Williams RS, Archer D, **Chegini N**. (2004) Doxycycline inhibits the expression of MMP and TNF-a in human endometrial stromal and epithelial cells: potential implication in dysfunctional uterine bleeding. Soc for Gynecol Invest 51th Annual Meeting, 2004 (accepted for presentation).

Exhibit B

[Frontiers in Bioscience 7, e91-115, April 1, 2002]

PERITONEAL MOLECULAR ENVIRONMENT, ADHESION FORMATION AND CLINICAL IMPLICATION

Nasser Chegini

Division of Reproductive Endocrinology and Infertility, Institute for Wound Research, Department of Obstetrics and Gynecology, University of Florida, Gainesville, FL, 32610

TABLE OF CONTENTS

1. ABSTRACT

Whether induced by infection, inflammation, ischemia, and/or surgical injury, peritoneal adhesions are the leading cause of pelvic pain, bowel obstruction and infertility. It is clear that while postsurgical peritoneal wounds heal without adhesions in some patients, others develop severe scarring from seemingly equal procedures; in addition, in the same patient, adhesions can develop at one surgical site and not in another. The mechanisms underlying the predisposition to form adhesions as well as their site specificity are completely unknown. However, a large number of intraperitoneal surgical procedures are performed each day in the USA, and thus many patients are at risk of developing postoperative adhesions. Therefore, understanding of adhesion formation at the molecular level is essential and in the absence of such information, attempts to prevent patients from developing adhesions will remain an empirical process. The unprecedented advancement in molecular biology during the past decade has led to the identification of many biologically active molecules with the potential of regulating inflammatory and immune responses, angiogenesis and tissue remodeling, events that are central to normal peritoneal wound healing and adhesion formation. Although, the insight into their importance in the development of tissue fibrosis has substantially increased, their major roles in peritoneal biological functions and adhesion formation remain at best speculative. This article reviews the clinical implications of adhesions and attempts to highlight some of the key molecules i.e. growth factors, cytokines, chemokines, proteases and extracellular matrix, that are recognized to regulate inflammation, fibrinolysis, angiogenesis, and tissue remodeling, events that are central to peritoneal wound repair and adhesion formation. Finally, the article discusses the potential application and site specific delivery of several active compounds that are developed to alter the local inflammatory and immune response i.e., cytokine/chemokine network, targeted gene delivery and development of a new generation of biomaterials to prevent adhesion formation. Such understanding of peritoneal biology not only assist us to better manage patients with adhesion, but also those with endometriosis and malignant diseases that affect the peritoneal cavity.

2. CLINICAL IMPLICATIONS

In humans, postoperative adhesion formation has been a problem since the advent of intra-abdominal manipulation. Even today, after immense improvement in surgical instrumentation and techniques as well as the development of various postsurgical devices to prevent

adhesion formation, intraperitoneal adhesions remain a major clinical concern. During the past two decades, clinical observations have consistently confirmed and estimated that between 60 to 100% of patients undergoing abdominal/pelvic surgery develop peritoneal adhesions. These observations have also revealed a direct proportional link between the frequency of surgery and the incidence of adhesion formation. Although postoperative adhesions are recognized as a consequence of surgery, patients with pelvic inflammation or endometriosis also are at risk of developing adhesions. For this reason, common use of laparoscopic intervention in various abdominal and pelvic surgical procedures has led to the belief that such an approach is less likely to cause adhesions with lower postoperative morbidity compared with laparatomy. Laparoscopic surgery often is more precise than open surgery, causing less tissue drying and more localized tissue trauma. However, the difference between the incidence of postoperative adhesion formation caused by laparatomy versus laparoscopic procedures is, at best, controversial.

While most of the earlier studies have been conducted in female infertility patients, recent studies showed that 94% of women without infertility and men who had undergone colectomy developed adhesions immediately beneath the midline abdominal incision at second-look (1-8). The most recent epidemiological studies, which examined a ten year follow-up of over 54,000 patients' medical records from the Scottish National Health Service Registrar Database, have clearly pointed out both the immediate and long term scope of peritoneal adhesion associated morbidity following gynecological and lower abdominal surgeries (2,3). These studies have concluded that 30 to 35% of all hospital readmissions were associated with potential adhesion associated complications, of which 4.5 to 5.1% were directly related to adhesions. Patients who underwent gynecological surgery involving ovaries and fallopian tubes were even at the highest relative risk of hospital readmission for complication directly related to adhesions, with an overall rate of readmission of 80 to 100 per 100 initial procedures, with 15% occurring during the first year and continued over the next 10 years. A similar trend was also found in hospital readmission of patients who had undergone gastrointestinal surgery, with an overall rate 2.5 times higher than other lower abdominal surgical procedures. Another 22.1% of patients required additional surgery for reasons that were possibly related to adhesions (3). Peritoneal adhesions affecting the uterus, fallopian tubes, or ovaries have been shown to account for approximately 20% of all infertility, a problem that is experienced by 10% of reproductive age women (1). Collectively, adhesions are a key initiating cause of infertility, chronic abdominal/pelvic pain and bowel obstruction, and they impair the organs' unique physiological functions. Although controversial, the presence of abdominal-pelvic pain has often been attributed to adhesions, which are estimated to be a contributing factor in up to 30% of women with chronic pain (1). Extensive adhesions can also lead to serious complications for peritoneal dialysis patients or those who require intraperitoneal drugs or nutrition. Even for patients who do not suffer from adhesion-associated complications, the presence of adhesions in re-operative procedures can prolong operative time and increase the risk of intraoperative/postoperative complications due to injuries to the bowel, bladder, blood vessels or other sites (1-8). Failure to identify and acknowledge the scope of adhesion-associated problems may be a contributing factor that has hampered our goal of developing measures to prevent adhesions, and is due in large part to ethical and logistical restraints to the performance of second-look operations to assess the extent of postoperative adhesion development.

The recognition that adhesions contribute to the above clinical problems has generated a great deal of interest in development of creative techniques or products to prevent postoperative adhesion development (5-8). Despite continued efforts and advancements, these measures have been found to be ineffective; rather, at best, they only reduce the incidence of adhesion formation. Even currently available devices, i.e. bio-absorbable membranes, gels, and coating solutions, as well as materials presently undergoing clinical trials, are formulated such that they have a casual relationship with the ultimate goal of serving as an ideal tool to prevent postoperative adhesion development. In addition to a significant clinical burden associated with surgical and medical work loads, the economic impact of adhesion related to medical expenses, currently estimated to be around 2-4 billon dollars annually in the United States alone (1-8), as well as the indirect costs associated with reduced business efficiency or time lost from work, make finding a successful adhesion prevention strategy an urgent necessity. However, to reach this goal we must first increase our understanding of the peritoneal wound environment at the molecular level, which will allow the development of devices that far exceed the need of satisfying the basic biocompatibility with the surrounding environment. Most of our current knowledge of peritoneal wound healing and adhesion formation derives from analogous studies of dermal wound healing and scar tissue formation. Such comparisons have been instrumental in increasing our overall knowledge of the peritoneal wound molecular environment, since peritoneal adhesion formation is a defective wound healing process that is similar to dermal scar formation. However, there are reasons to address the issue of peritoneal wound healing and adhesion formation in a different context in order to identify those differences that may aid us to more effectively reach our ultimate goal. We still poorly understand why adhesions form more frequently in an individual tissue and/or patient than in others. Although improved surgical techniques and limited manipulation of organs during surgical procedures are probably important in preventing adhesions, the post-genomic era with its wealth of information may be useful in answering this question at the molecular level. In addition, the development of superior postsurgical devices that have biocompatibility with their surrounding environment and carrying wound modifying agents could improve the management of adhesion development. In this review, we attempt to highlight some of the key molecules recognized to modulate the outcome of wound healing and scar tissue formation and discuss potential avenues that could be utilized to locally alter their expression to reach an adhesion-free peritoneal environment.

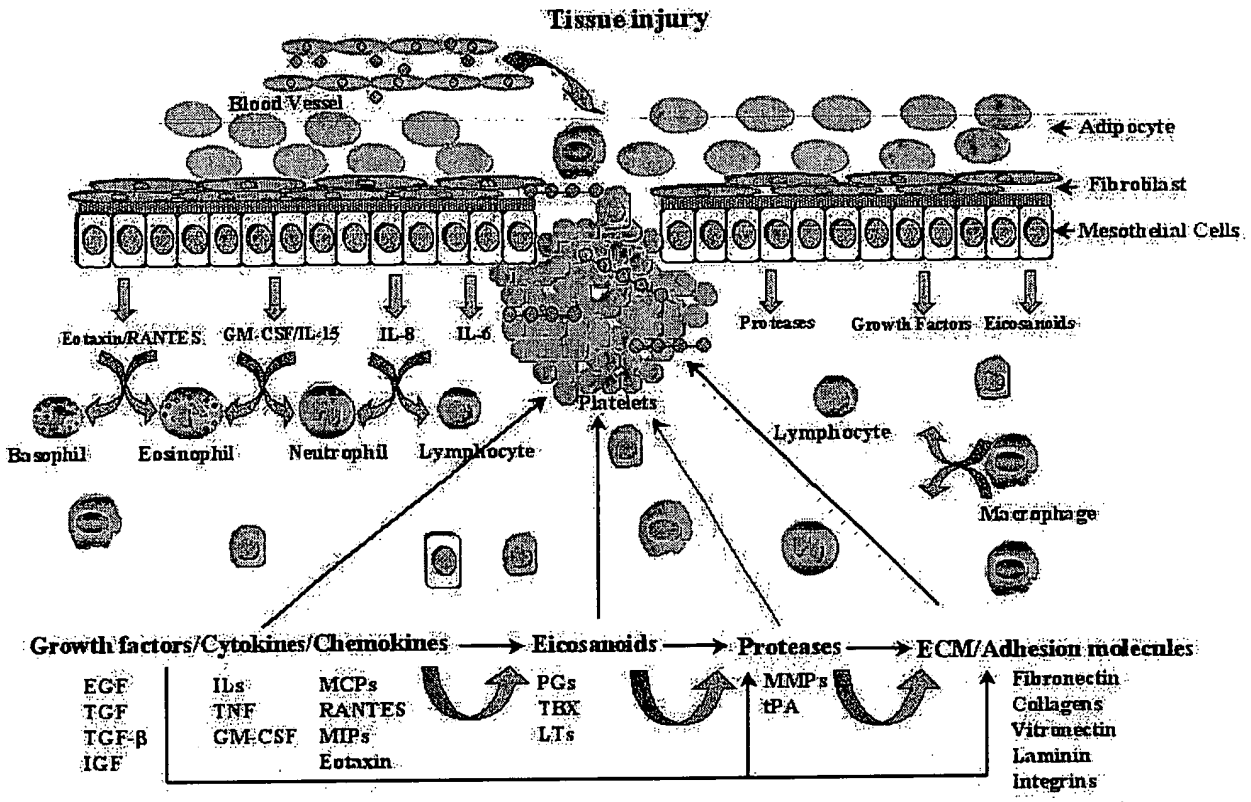


Figure 1. Schematic representation of early events following peritoneal injury and involvement of a number of cytokines, chemokines, growth factors, eicosanoids, proteases and adhesion molecules that regulate blood clot formation, inflammation, cell migration involving leukocytes, macrophages, and fibroblasts to initiate the wound repair.

3. MOLECULAR EVENTS IN PERITONEAL ADHESION FORMATION

The serosal surface of intraperitoneal organs and the parietal peritoneum is composed of mesothelial cells. Taken together, these surfaces comprise the largest surface area in the body that almost equals that of the skin. Peritoneal inflammation, ischemia, infection, and surgically-induced tissue trauma can easily damage or permanently remove these cells, exposing the submesothelial connective tissue (Figures 1 and 2). The onset of cellular and tissue injury is associated with remarkable morphological and biochemical alterations which promote repair of the defective region. However, when this process is unregulated, the outcome of wound healing is associated with excessive tissue or scar formation (9). In contrast, fetal wounds heal with near-perfect regeneration or scarless healing (9). The regulating mechanism underlying fetal tissue regeneration is unknown, although the extent of trauma and degree of inflammation are considered important elements. Interestingly, this phenomenon appears to apply only to dermal wound healing, because injury to other fetal tissues, including intraperitoneal organs, results in scarring similar to that which occurs in adult tissues. In addition, aging slows the rate of dermal wound healing, while peritoneal wounds do not appear to follow this trend.

The overlapping and dynamic processes that lead to peritoneal wound healing include inflammatory response, cell growth and differentiation, angiogenesis, extracellular matrix turnover, tissue remodeling, and apoptosis. While many phases of peritoneal wound repair and specific mechanisms regulating these activities resemble dermal wound healing, two fundamental differences must be taken into consideration. First, dermal injuries heal inward from the edges such that the rate of healing is dependent on the size of the lesion. On the other hand, peritoneal wounds are thought to heal by the differentiation of underlying progenitor cells, the movement of mesothelial cells from the edge of the wound towards the center, and by seeding of the wound with mesothelial cells detached from other regions, such that healing occurs simultaneously throughout the lesion and independent of the surface area of the injury (10). Second, peritoneal wounds are continuously exposed to many substances that are synthesized and released by mesothelial, inflammatory and immune cells in peritoneal fluid and various other cell types within the wound. Therefore, direct and indirect autocrine/paracrine feedback regulation initiated by local expression of these molecules is an important component and determinant of the outcome of peritoneal healing and adhesion formation.

The identity and precise nature of the molecules that are involved in peritoneal repair processes are not yet known. However, emerging evidence from studies examining peritoneal wound and peritoneal fluid during healing and comparative analogy with dermal wound

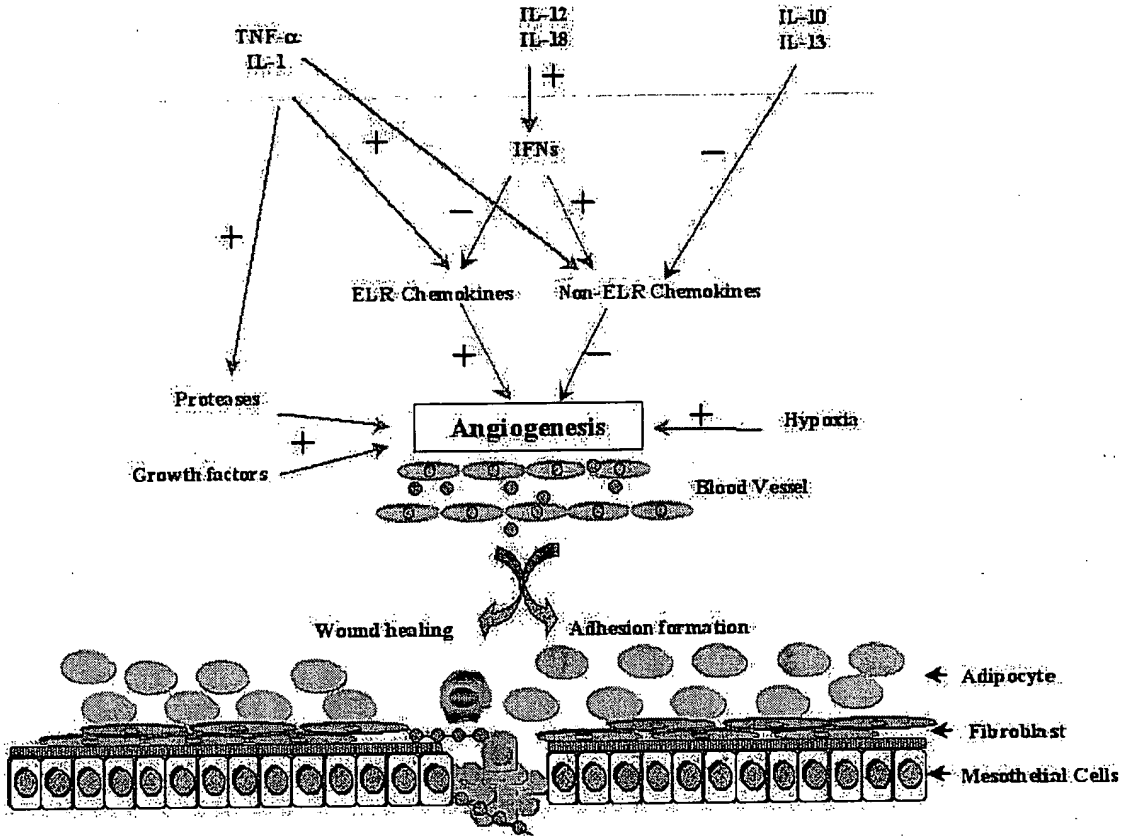


Figure 2. Schematic representation and involvement of a number of cytokines, chemokines and growth factors that regulate angiogenesis that ultimately leads to either peritoneal wound healing or adhesion formation.

healing has led to the hypothesis that local expression of autocrine/paracrine growth factors, cytokines, chemokines, proteases, adhesion molecules and the extracellular matrix, etc. plays a critical role in these events. As evidenced by different degrees of peritoneal tissue repair, the expression of these molecules and their biological signals must be optimal, precise, and synchronized. The correct and timely progression of expression and interaction among these signals appear to provide an optimal environment for near perfect tissue repair. In contrast, the aberrant function of some of these factors seems to be responsible for inappropriate wound healing processes affecting the inflammatory response, cellular migration, proliferation, angiogenesis, and tissue remodeling that result in peritoneal adhesions.

3.1. Inflammatory Mediators and Adhesion Formation

Most of our understanding of the process of peritoneal inflammation and its key regulators has been accumulated from clinical and basic science research on pelvic inflammatory disease and peritoneal infection in peritoneal dialysis patients (11). Peritoneal dialysis is performed in a substantial number of patients annually and is also the leading cause of adhesion formation (11). These studies have led to recognition of an array of molecules that are established as key regulators of peritoneal host defense mechanisms and functional activity generated to heal the defected area in response to infection, inflammation or cellular/tissue injury (10-12). The inflammatory and immune cells that reside or migrate into the peritoneal cavity, mesothelial cells lining the visceral and parietal peritoneum, fibroblasts which reside within the submesothelial tissue, and their secretory products are the key regulators of the peritoneal response to these events (10-12). The individual and combined actions of these molecules are to initiate, amplify, and control many molecular events that ultimately lead either to resolution of the inflammatory response, peritoneal wound repair, or adhesion formation (Figures 1 & 2).

In the case of peritoneal injury, coagulation and platelet aggregation are initiated to prevent excessive blood loss. Cell surface activation of Hagemann factor, a tissue procoagulant factor released from damaged cells, as well as the activation of surface membrane coagulation factors and phospholipids, expressed by activated platelets and vascular endothelial cells, initiate these events and promote the healing of peritoneal defects (13-15). At the same time, as a requirement for initiation of tissue repair, the clots must begin to lyse. Both clot formation and degradation are regulated by many intrinsic substrates including eicosanoids such as prostacyclin, thromboxane, leukotrienes, antithrombin III, protein C, plasminogen activators, and plasminogen activator inhibitors (13-21).

The activities of these substances are regulated by the local release of various soluble factors produced by platelets and, later in the process, by infiltrating and activated inflammatory and immune cells. Platelets release a network of highly active substances into the wound environment, some with adhesive properties that act as ligands for platelet aggregation, e.g. fibrinogen, fibronectin, thrombospondin, and von Willebrand factor VIII. Von Willebrand factor VIII also mediates platelet adhesion to fibrillar collagens through interaction with integrin and subsequent platelet activation (14-18). Activated platelets also release leukocyte chemotactic factors, platelet derived growth factor (PDGF), transforming growth factor alpha (TGF-α), heparin binding epidermal growth factor (HB-EGF), transforming growth factor beta (TGF-β), etc. (22-27). The fibrin clot, which provides an early form of extracellular matrix (ECM), retains many of these factors that potentiate the migration of inflammatory cells into the wound (Figures 1 & 2).

The induction of a local inflammatory reaction due to infiltration of peripheral blood neutrophils and monocytes is an important feature of soft tissue repair, and animals depleted of macrophages have defective tissue debridement, fibroblast proliferation, and wound repair (9). Macrophage recruitment into the tissues is further sustained in part by platelet-derived cytokines as well as other chemoattractant factors. These factors include fibrinopeptides cleaved from fibrinogen by thrombin, degradation products of fibrin produced by plasmin, platelet factor 4 (PF4), eicosanoids (LTB_4, LTC_4, and PGE_2), and platelet-activating factor (PAF) released from endothelial cells or activated neutrophils (28). The recruitment of inflammatory cells into the wound is also facilitated by adhesive molecules such as fibrin, fibronectin, and vitronectin that are present in the fibrin clot (provisional matrix), as well as integrins that recognize these molecules (9,19-21). The infiltrating monocytes that become activated and differentiate into macrophages are a major source of growth factors, cytokines, chemokines, eicosanoids, and proteases (22,25-27). In addition, peritoneal macrophages, neutrophils, T cells, mast cells, and mesothelial cells are the sources of many of these molecules. Collectively, these molecules, either individually or through their interactions, regulate the peritoneal wound as it proceeds through a degradative phase into a reparative stage that ultimately results in peritoneal tissue repair.

Many of the cytokines and chemokines released into the wound regulate the production of eicosanoids and proteases that are essential factors in the coagulation cascade and inflammatory response (19-29). The production of cytokine-induced eicosanoids through the cyclooxygenase pathway appears to antagonize, while products of lipoxygenase pathway mediate or amplify the effect of cytokine action (30,31). The stimulatory actions of TGF-β, EGF, and TNF-α on fibroblasts, a key cell type in adhesion formation, are augmented by cyclooxygenase inhibitors and reduced by the presence of PGE_2, PGE_1, and PGI_2 (9,22,24,32). In addition, eicosanoids act as intracellular mediators of cytokine and growth factor actions in various cell types. Elevated production of eicosanoids and their receptors has been associated with an increased incidence of adhesion formation, and their inhibition through the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, indomethacin and meclofenamate, has proven effective in reducing adhesion formation in animal models. Although limited studies performed to establish efficacy in humans have had mixed results (33-35), evidence generated from patients with peritoneal inflammation due to peritoneal dialysis, animal models of peritoneal inflammation, and in vitro studies, support the use of agents to suppress peritoneal eicosanoid production in the peritoneal cavity following infection or surgical trauma. Intraperitoneal (IP) administration of indomethacin in continuous ambulatory peritoneal dialysis patients during peritonitis has been reported to decrease the intrinsic permeability of the peritoneum to macromolecules without effectively altering other peritoneal functional parameters (36). In addition, IP administration of indomethacin following peritoneal bacterial challenge enhances leukocyte migration and peritoneal permeability to protein and dialysate concentrations of PGE2, PGF1α and IL-8. Such enhancement occurs without affecting the transperitoneal migration of leukocytes or the production of IL-8, a potent inflammatory cytokine (37). Leukotrienes, which are potent chemoattractant factors for neutrophils, macrophages, and eosinophils have been implicated in the pathogenesis of a variety of inflammatory processes. Surgically-induced peritoneal injury results in upregulation of prostaglandin, thromboxine, 5-lipoxygenase, and receptors for LTB-4, LTC-4 and LTD-4 throughout healing and peritoneal adhesion formation (33,34). LTB-4 mediates its action through a G protein-coupled receptor, the LTB-4 receptor (BLTR), and targeted gene disruption of BLTR has further implicated LTB-4 as a key factor in leukocyte activation. Despite a functional redundancy with other chemoattractant-receptors, it is apparent that LTB-4 and BLTR are central to the recruitment and/or retention of leukocytes, particularly eosinophils, to the inflamed peritoneum (38). BLTR deficiency did not result in any apparent abnormalities and peritoneal neutrophils displayed normal responses to the inflammatory mediators such as PAF; however, neutrophil influx in response to arachidonic acid was significantly reduced following the induction of peritonitis in BLTR deficient mice. Surprisingly, female mice deficient in BLTR displayed selective survival relative to males in response to PAF-induced anaphylaxis (39). This finding may be due, in part, to the protective action of ovarian steroids.

3.2. The Role of Chemokines and Cytokines

Chemokines are small polypeptides divided into several subgroups according to the spatial arrangement of the first two cysteine residues that include CXC or α, CC or β, C or γ and CX3C or δ chemokine (40). Chemokines have a potent chemoattractant activity for leukocytes and regulate inflammation, angiogenesis, hematopoesis, and host response to infection. They mediate their biological activities through a distinct family of G-protein-coupled receptors that have been identified on various cells types (41). The two most prominent chemokines are the CC

which include monocyte chemoattractant protein-1 (MCP-1), MCP-5, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, eotaxin, regulated upon activation, normal T-cell-expressed and secreted (RANTES), C10, stromal cell-derived factor 1 (SDF-1) and CXC such as IFN-inducible protein-10 (IP-10), MIP-2, KC and growth-related oncogene, Gro-*a* (40,41).

Recent in vitro and in vivo studies also point to the critical role for chemokines in peritoneal biology, specifically, the inflammatory response that often leads to adhesion formation (11). Plasma-derived fibrinogen released in response to endothelial cell retraction at sites of inflammation has been shown to induce peritoneal macrophage expression and release of several chemokines, including MIP-1α, MIP-1β, MIP-2, and MCP-1 (42). MCP-1 in turn stimulates peritoneal macrophage LTB-4 production. The LTB-4 receptor antagonist, CP-105-696, inhibits peritonitis-induced recruitment of neutrophils and macrophages, accompanied by a reduction in peritoneal MCP-1 expression and survival of mice (43). These results suggest that induction of chemokines following the initiation of peritoneal injury occurs directly or indirectly through the production of LTB-4, and that chemokines mediate the recruitment of neutrophils to the site of injury (43). Induced peritoneal macrophages are also associated with the induction of chemokines such as RANTES, MCP-1 and MIP-1α (44). IP administration of MCP-1 promotes chemotaxis of specific leukocyte populations predominately consisting of granulocytes and macrophages, while eotaxin selectively enhances eosinophils, and MIG induces T cell migration. Administration of methotrexate, piroxicam or dexamethasone inhibited cellular migration, and MCP-1-mediated trafficking was impaired by treatment with anti-MCP-1 antibody or IL-10, a cytokine with anti-inflammatory properties (44). Interestingly, MIP-2 expression was found to be limited to localized inflamed regions, independent of the expression of proinflammatory cytokines such as TNFα or IL-1β production (45). IFN-γ treatment selectively inhibited LPS-induced MCP-1, MIP-1α, MIP-1β and MIP-2, but induced IP-10 and MCP-5 expression (46). Peritoneal B lymphocytes which express the SDF-1 receptor accumulate and proliferate in response to SDF-1 chemotactic and growth-promoting action, and IP administration of neutralizing SDF-1 antibody significantly decreased their numbers, suggesting that peritoneal derived SDF-1 may play a role in the peritoneal migration of B cells (47). In addition, SDF-1 reversed the antigen-induced T-cell recruitment into the peritoneal cavity, a phenomenon described as a movement away from a chemokine, which represents a previously unknown mechanism regulating the localization of mature T cells (48). IL-3, IL-4, GM-CSF, IL-10 and IL-13, cytokines that are expressed by mesothelial cells and are present in peritoneal fluid, induce macrophage expression of C10, a CC chemokine that regulates the chronic stage of host defense reaction. In addition, MCP-1 and MIP-1α expression is induced by IL-3 and GM-CSF, and inhibited by IL-4 or IFN-γ. In contrast MCP-1 and MIP-1α inhibit IL-3- and GM-CSF-induced C-10 expression. The peritoneal level of C10 is increased following the induction of peritonitis, and IP administration of C-10 induced a rapid production of TNF-α and MCP-1, and a later increase in IL-13 levels, which were negatively impacted following IP anti-C10 antiserum therapy (49,50).

IL-13 is a Th2 cytokine with a potent anti-inflammatory activity. Induction of peritonitis in mice increased tissue expression of IL-13 in liver, lung, and kidney, but not in peritoneal fluid or in serum, and the inhibition of IL-13 with anti-IL-13 antibodies resulted in a decreased rate of survival (51). Interestingly, treatment with anti-IL-13 antibodies did not alter peritoneal leukocyte infiltration and bacterial load, but it did increase the influx of neutrophils into these tissues, as well as the expression of MIP-2, KC, MIP-1α and TNF-α (51). These finding suggest that endogenous IL-13 mediates its protective action through suppression of tissue expression of inflammatory cytokines/chemokines. Another cytokine with anti-inflammatory properties is IL-10, which effectively inhibits the expression of MCP-1, MCP-5, MIP-1α, MIP-1β, MIP-2, IP-10, KC and RANTES. In addition, IFN-γ selectively induces IP-10 and MCP-5 expression, but inhibits LPS-induced MCP-1, MIP-1α, MIP-1β, MIP-2 and KC (46). Human peritoneal fibroblasts constitutively express MCP-1 and IL-8 and their expression is augmented by the pro-inflammatory cytokines, IL-1β and TNF-α, and by peritoneal macrophage-conditioned medium (52). These effects were partly due to the presence of IL-1β, since co-treatment with the IL-1 receptor antagonist reduced their production (52). IL-1β, TNF-α, IFN-γ or their combinations also modulate the expression of MCP-1, RANTES, IP-10 and GRO*a* by mesothelial cells, by inducing GRO*a* and IP-10 expression, and they augment constitutive production of MCP-1, with limited effect on RANTES expression (53). Furthermore, GRO*a* (8-73), a CXC chemokine receptor antagonist, prevented MIP-2 and KC-induced neutrophil migration (54). Prior treatment with GRO*a* (8-73) or an analogue of PF4- (9-70) resulted in inhibition of leukocyte infiltration into the peritoneal cavity in response to MIP-2. Moreover, GRO*a* (8-73) treatment effectively inhibited TNF-α-, IL-1β- or LPS-induced leukocyte recruitment. These observations point out the importance of complex interactions among cytokines and chemokines in peritoneal inflammatory and immune responses (50), and suggest that their modulation in the peritoneal environment might influence peritoneal inflammation, healing, and adhesion formation.

Hyaluronan (HA) and its derivatives are extensively used in fabrication of biodegradable materials such as membranes, gels, and solutions to prevent adhesion formation (6-8). Degradation products of HA often accumulate at sites of inflammation, and experimentally generated HA fragments have been shown to enhance the expression of MCP-1 and IL-8 in human mesothelial cells (55). Therefore, increased HA levels in the peritoneal cavity of peritoneal dialysis patients as well as the byproducts of biodegradable HA-based devices may alter the local production of these and other chemokines, resulting in prolonged inflammation (55). In addition, neutrophil elastase produced during peritoneal inflammation and at the site of tissue injury enhance the production of MCP-1 by peritoneal macrophages, which was inhibited by serine protease inhibitor,

phenylmethylsulfonyl fluoride (56). This suggests that, in addition to degradation of ECM, proteases may stimulate the release of chemokine production by macrophages, leading to impairment of peritoneal healing and adhesion formation.

The resolution of the inflammatory reaction, which begins with reduced neutrophil infiltration, is also essential for the repair process to proceed. Tissue neutrophils are entrapped within the clot and desiccated tissue, where they become apoptotic and are phagocytosed by macrophages (57-61). A number of cytokines, including GM-CSF, IL-1β, TNF-α, and IFN-γ that are expressed by the mesothelial cells and other wound cells and are present in peritoneal fluid, promote the uptake of neutrophils by macrophages (57-61). The mechanism involved in the clearance of macrophages after the completion of their function in the inflammatory reaction has not been defined, although macrophages can undergo apoptosis under in vitro conditions (25,26,57-61). The continued and appropriate expression of cytokines, chemokines and their receptors is critical to the resolution of peritoneal inflammation and the transition from an inflammatory to a reparative environment. Because peritoneal mesothelial cells and fibroblasts interact with peritoneal inflammatory and immune cells, identification of specific chemotactic, pro-inflammatory, and anti-inflammatory cytokines and chemokines which regulate their interactions may allow to design better strategies to prevent the development of adhesions. Such studies would further increase our understanding of other biological processes that result in either uncomplicated repair or the development of adhesions, involving cellular migration, proliferation, and differentiation of several cell types that promote angiogenesis, tissue remodeling and synthesis and deposition of ECM.

4. ANGIOGENESIS AND PERITONEAL ADHESION

In the healthy adult, angiogenesis is restricted to the corpus luteum, placenta, endometrium during the menstrual cycle, and during wound healing. Angiogenesis is a self-limited and strictly regulated process that occurs in a sequential manner. It involves degradation of the vascular basement membrane and interstitial matrix by endothelial cells, migration and proliferation of endothelial cells, and finally tubulogenesis and formation of capillary loops (62-66). The production of proteolytic enzymes in response to angiogenic factors is fundamental to angiogenesis, not only for the degradation of perivascular matrix and tissue stroma, but also for the migration and proliferation of endothelial cells. During angiogenesis, the initial migration and proliferation of endothelial cells occurs in a fibronectin rich ECM, whereas vascular maturation, which takes place at later stages, is laminin rich (9). These processes also involve integrins, the essential components of ECM-cell and cell-cell interactions that promote cell migration, gene expression, cell differentiation and other cellular activities (20,21,62-66). At the initial stage of angiogenesis, the induction of proteases such as matrix metalloproteinases (MMPs) and serine proteases (fibrinolytic system; plasminogen activators) in endothelial cells is necessary to degrade components of the ECM, including fibronectin and laminin (14-21,62,67-69). However, these proteases are produced in inactive forms and must become activated to initiate their local actions. Proteolytic activities of these enzymes are regulated by naturally occurring physiological inhibitors, tissue inhibitor of MMPs (TIMPs) and plasminogen activator inhibitors (PAIs) (14-21,67-71). Cytokines and growth factors, such as IL-1, IL-8, TNF-α, GM-CSF, VEGF, FGFs, EGF, TGF-α, TGF-β, PDGF, and IGF-I, are considered to be angiogenic enhancing factors due to their ability to, 1) regulate the expression of MMPs, the fibrinolytic system and their inhibitors, and 2) to modulate endothelial cell proliferation and migration (14-21,62-74). MMPs, tPA, uPA, TIMPs and PAIs are expressed in parietal peritoneum, peritoneal mesothelial cells and adhesion fibroblasts, and similar to other systems, their expression is regulated by various cytokines and growth factors (13,16-22,62-78). Human peritoneal capillaries and arteriole endothelial cells express VEGF and other angiogenic factors (79) that may regulate the proteolytic enzymes and their inhibitors (Figures 2 & 3).

Since VEGF plays a key role in coagulation, fibrinolytic, and angiogenic activities, it is considered to be a critical cytokine in the development of peritoneal adhesions. Four species of mRNA encoding VEGFs arising from alternative splicing have been identified (VEGF 188, 165, 145 and 120) of which VEGF188, 165 and 120 are expressed in peritoneal wounds. Up-regulated expression of VEGF188 and 120 occurs during the early stages of peritoneal healing, and down-regulation of VEGF165 expression has been observed 24 to 48 hrs following injury (80). Hypoxia, a condition that promotes peritoneal adhesion, alters the expression of several cytokines, chemokines and eicosanoids in the wound, including the expression of VEGF and TGF-β, shown to cause tissue fibrosis (81). The peritoneal mesothelial and vascular endothelial cells of blood vessels, which supply peritoneal adhesions, express both VEGF and FGF-2, supporting a role for these cytokines in mediating peritoneal angiogenesis during adhesion formation (79). Although the expression of FGFs has been documented in a wide variety of cells and tissues, including monocytes/macrophages, T lymphocytes, vascular endothelial cells, fibroblasts, keritonocyte, and mesothelial cells (71-73,79,80), a profile of their expression and biological activities during peritoneal wound healing and adhesion formation awaits investigation. FGF-1 and FGF-2 lack the classical secretory signal peptides, and their detection in the ECM or peritoneal fluid is of unknown cellular origin. However, they are released as a result of mechanically-induced injury (73,74). Peritoneal injury could serve as a mechanism to release FGF from mesothelial cells and/or infiltrating inflammatory cells, which along with VEGF and EGF/TGF-α act as angiogenic factors as has been shown in omental microvascular endothelial cells. Delayed wound angiogenesis and healing in aged animals have been associated with alteration of FGF-2 and VEGF expression. Subcutaneous administration of rFGF induced less capillary growth into matrigel in aged mice than in young mice (82). These findings further suggest that a decline in angiogenic

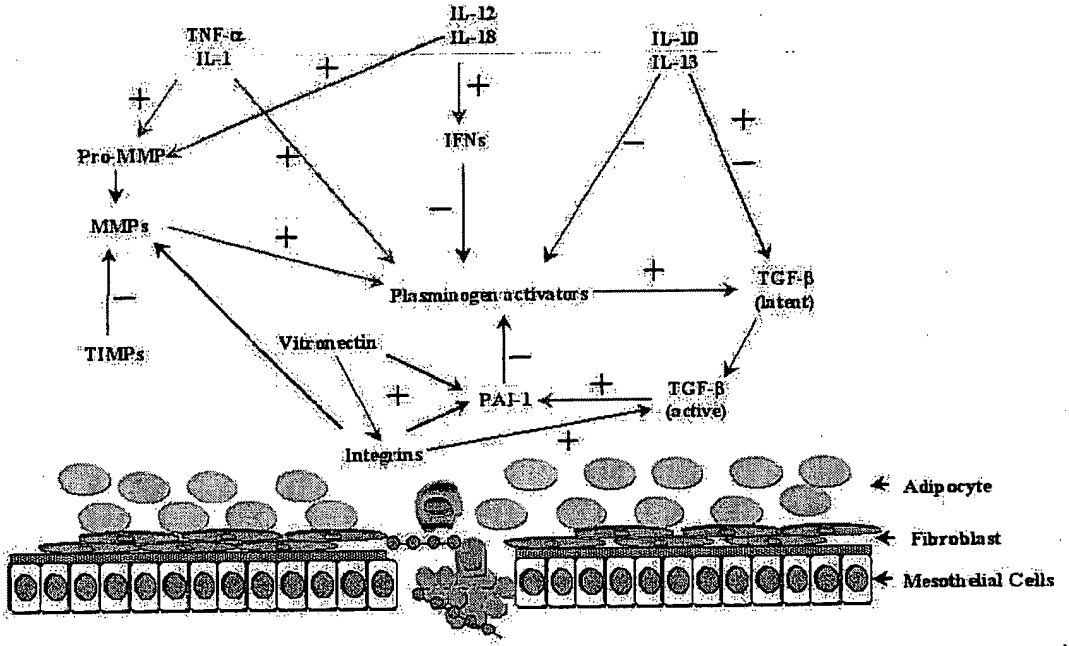


Figure 3. Schematic representation of and involvement of a number of cytokines, growth factors, proteases and adhesion molecules that regulate fibrinolysis resulting in either peritoneal wound healing and/or adhesion formation.

growth factor production and alteration in endothelial responsiveness may account for delayed wound angiogenesis and healing (82).

The angiogenic property of these growth factors is reflected in their ability to alter the expression of PA (uPA and tPA) and PAI-1 in microvascular endothelial cells and stimulate their invasion (16,17,66,71,73). For instance, VEGF stimulates von Willebrand and tissue factors in endothelial cells, and VEGF, FGF, EGF, and TGF-α either alone or through synergistic interactions, stimulate PA expression that converts TGF-β from its latent to active form (16,17,22,63,71,73). The active form of TGF-β inhibits PA expression, providing a feedback loop that modulates FGF, VEGF, TGF-α and PA expression. FGF-2 and TGF-β also have an opposing effect on PA activity; FGF acts as a potent inducer of uPA expression with a relatively modest effect on PAI-I synthesis, whereas TGF-β downregulates uPA and upregulates PAI-I synthesis (22,63). Kertitonocyte growth factor (KGF or FGF-7), a highly active mitogen for epithelial cells, also stimulates uPA expression (63,71,73,74). Several cytokines and chemokines also regulate the expression of fibrinolytic system, including several interleukins, M-CSF, GM-CSF and MCP-1 (11,12,65,66).

Angiogenesis is also dependent on the balance between angiogenic factors and their inhibitors. Among the angiogenic suppressors are cytokines, such as TGF-β, TNF-α, IFNs, and several other agents (71,83-85). These include collagen synthesis modifiers, protamine (an arginine rich protein that inhibits the mitogenic action of FGF), cyclosporin, PF-4, and HA (although its degradative products may be angiogenic), thrombospondin, which is released by platelets and is present around mature quiescent vessels, but absent from actively growing sprouts, and angiostatin, a tumor suppressor factor with angiogenic inhibiting property and considerable homology with plasminogen and hepatocyte growth factor (HGF), another angiogenic factor (83). In addition, an endogenous estrogen metabolite has been shown to inhibit angiogenesis in vivo and prevents endothelial cell proliferation and migration in vitro through a mechanism involving the induction of uPA (83,85). Vascular endothelial cells, including those in the peritoneum, contain receptors for ovarian steroids, suggesting that steroids can potentially regulate vascular activities that result in peritoneal healing and adhesion formation (79). Although many aspects of neovascularization in the peritoneum await investigation, the expression of these factors in peritoneal tissue and their presence in the peritoneal fluid imply that both direct and indirect action of these molecules can alter angiogenesis during peritoneal healing and adhesion formation. A number of anti-angiogenic factors are currently under intense investigation and results from these studies have revealed that factors which initiate, control and terminate the multi-stage process of angiogenesis may be useful in interfering with angiogenesis. Among these are compounds that inhibit angiogenic growth factors (IFN-α, suramin, and analogues) or their receptors (SU6668, SU5416), as well as endogenous inhibitors of angiogenesis (endostatin, IL-12), which are currently being tested for the treatment of a variety of disorders in clinical trials (86). Preoperative treatment with TNP-470, a potent endothelial cell inhibitor, has been reported to reduce vessel ingrowth, and associated sustained reduction in adhesion formation (87). In addition,

protease inhibitors, inhibitors of the endothelial cell proliferation, suppressors of angiogenic growth factors, and cytokines are also being investigated for their potential in controlling angiogenesis. These compounds may be useful as potential agents in the management of peritoneal wound healing by limiting tissue fibrosis such as adhesions.

5. PERITONEAL TISSUE REPAIR

The repair of denuded parietal peritoneum begins immediately after the injury. Various growth factors, cytokines, and chemokines, along with their specific receptors or binding proteins, appear to have the potential property of orchestrating such rapid and dramatic tissue reorganization. The expression of many of these cytokines and growth factors, and a limited number of chemokines, have been detected in parietal peritoneum and adhesions. Although direct evidence to link any of these molecules to normal physiological and/or pathophysiological processes which affects the outcome of wound repair and scar tissue formation is lacking, their local expression during the wound healing suggests their importance in various peritoneal biological activities. Although available information regarding the involvement of cytokines and chemokines in cellular activities of peritoneal mesothelial cells and adhesion fibroblasts is limited, the receptor signaling pathways that mediate their intracellular biological activities remain largely unknown.

5.1. Role of growth factors

EGF was the first growth factor to be identified during peritoneal wound healing and adhesion formation, followed by characterization of other members of the EGF family including TGF-α and HB-EGF and their common receptor, the EGF receptor (88-90). Studies of the expression and cellular distribution of EGF, TGF-α and HB-EGF, and their receptors, in parietal peritoneum and adhesions revealed a widespread association with various wound cells. These data suggest that the EGF family of growth factors, along with their receptors, might potentially affect a wide range of activities during peritoneal healing. Consistent with this possibility, EGF was found to act as a mitogenic factor for mesothelial and adhesion fibroblasts, which are synergistically enhanced by VEGF, IGF and PDGF (91). Although VEGF is expressed by mesothelial cells and during surgically-induced adhesion formation (79,80), its impact on the outcome of peritoneal adhesion formation is not known. However, anti-VEGF neutralizing antibody lowered VEGF expression in wound fluid and reduced vascularization without affecting granulation tissue formation (81,92), further implicating VEGF in angiogenesis. IGFs and their binding proteins (IGFBPs), which regulate IGF-I availability, are present in the circulation and in tissue fluids in association with IGFBPs through high affinity binding (93). IGFs, IGF-BPs, and the IGF receptor are expressed throughout the period of peritoneal wound healing, with significant increases in their expression during adhesion formation (94). Interference with IGF-I activity, both systemically and intraperitoneally through hypophysectomy (resulting in low IGF-1 levels) or IP administration of IGFBP-4, have been shown to reduce the severity of postoperative peritoneal adhesions (95). IGFBP-3 binds the native fibrin clot, fibrinogen, and fibrin through its interaction with the heparin binding domain and in the presence of plasminogen. Such interactions may concentrate IGF-1 at wound sites, and upon its release binds to IGF receptors that are present on stromal cells migrating into the fibrin clot (96). Fibrin clot associated IGF and other growth factors can become accessible for local biological activities through the proteolytic action of various proteases that are present in the early phase of tissue injury. Neutrophil proteases, cathepsin G, and elastase, in addition to their functions as ECM-degrading enzymes, can potentially regulate IGFs and IGFBPs during inflammation and wound healing (97). IGF-I has a growth promoting activity for many cell types; however, similar to EGF, IGF-1 is not a particularly strong mitogen for adhesion fibroblasts or mesothelial cells, and requires interaction with other growth factors such as EGF and PDGF (91). The requirement for growth factor interactions is related to the ability of an individual growth factor to act either as a competence (PDGF) or progression (EGF and IGF) factor. PDGF, TGF-α, HB-EGF and IGF-1, which are expressed and released by platelets and macrophages, can potentially act in this manner and influence cell migration and proliferation at the earliest stages of peritoneal wound healing (23,24).

Peritoneal mesothelial cell-derived HB-EGF, through its receptors HER-1 and HER-4, and associated proteins, integrin α3β1, regulate mesothelial cell migration by increasing β1 integrin expression and adhesion to collagen type I (90). Platelet-derived extract, which contains various wound-activating factors, has been reported to increase αvβ3 integrin expression, and to promote angiogenesis, revascularization and granulation tissue formation (98). Mesothelial cells and the serosal tissue of parietal peritoneum and several intraperitoneal organ express several integrins, including αvβ3. Following tissue or cellular injury, these integrins could participate in promoting angiogenesis, mesothelial cell migration, re-epithelialization and/or adhesion formation by altering fibroblast migration (99-101). The pattern of integrin subunit expression has been reported to be identical on mesothelial cells in the anterior peritoneum and uterine serosal, suggesting a bi-directional communication between peritoneal exudate cells and connective tissue fibroblasts (101). This pattern of integrin expression on mesothelial cells could be important since integrin α4-deficient T lymphocytes cannot migrate properly during thioglycolate–induced peritoneal inflammatory response (102). Furthermore, intergins could activate latent TGF-β, a key factor involved in all phases of the tissue repair processes including granulation tissue formation and tissue fibrosis (21,22,103,104). TGF-β is secreted as latent protein complex and stored at the cell surface in association with ECM proteins that regulate its availability and activity. TGF-β must become activated before binding to the TGF-β receptor, but the mechanism(s) underlying the conversion of latent to active TGF-β in vivo remain unknown. However, under in vitro conditions transient acidification and heating, as well as treatments with plasmin, thrombospondin-1, and mannose 6-phosphate have also

been reported to activate latent TGF-β (21,22,103-106). Activated TGF-β regulates integrin, ECM, and proteases such as the fibrinolytic system, MMPs and their inhibitors (9,21,22,103-107). TGF-βs have multiple biological activities depending on the cell type and the specific microenvironment. TGF-β has both stimulatory and inhibitory effects on cell growth and proliferation, and their mitogenic activity has been reported to be indirect and due to the induction of growth factors such as PDGF and PDGF α receptor (21,23). TGF-βs also enhance the expression of the EGF receptor, and are synergised with EGF-induced gene expression including the induction of TGF-β1, but not TGF-β2 expression, which in turn decreases the number of high affinity EGF receptors. Moreover, TGF-β upregulates its own expression in a variety of cell types, including adhesion fibroblasts and mesothelial cells (21,108,109).

Overexpression of TGF-β1 has been implicated in a number of disorders involving fibrotic abnormalities including pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, and dermal scarring (22,104,110-112). Deletion of the TGF-β1 gene through homologous recombination suggests that the release of TGF-β1 from degranulating platelets or its secretion by infiltrating macrophages and fibroblasts are not critical either to initiation or the progression of tissue repair, and further suggests that endogenous TGF-β1 may actually increase inflammation and retard wound healing (111). In contrast, TGF-β1-/- Scid-/- mice demonstrated a major delay in wound healing that involved wound inflammation, cell proliferation, and maturation. Immuno-deficient Scid-/- mice that express TGF-β1 do not experience a delay in wound healing, suggesting that the immune cells and TGF-β1 affect compensatory pathways in wound healing, and that the delayed expression of TGF-β2 and TGF-β3 that occurs in the absence of TGF-β1 is responsible for the delayed wound healing. In addition to TGF-β1, these observations further imply a key role for TGF-β2 and/or β3 in wound healing (112). In particular, TGF-β3 significantly increased wound cellular proliferation, breaking strength, and collagen deposition, indicating the importance of TGF-β3 in wound healing (113).

Evidence that implicates TGF-β in peritoneal adhesion formation results from experiments showing the expression of TGF-β in parietal peritoneum, and the serosal surface of several peritoneal organs. Adhesions with mesothelial cells and adhesion fibroblasts are major sites of TGF-β expression, and elevated levels of TGF-β have been observed in adhesion tissues and in the peritoneal fluid of patients with adhesions (114-116), as well as in surgically induced-adhesion formation in animal models (117-123). Mice heterozygous for TGF-β1 (+/-) experienced significantly lower adhesions and expressed at least two fold lower TGF-β1 protein in their peritoneal fluid compared with wild type (+/+) animals (122). Furthermore, postoperative peritoneal administration of TGF-β has been shown to increase adhesion formation, while neutralizing antibodies directed against TGF-βs reduced the incidence of adhesion formation (121,122). TGF-β1 and TGF-β3 are expressed in serosal tissue of parietal peritoneum, the uterus, oviducts, ovaries, omenta, the large and small bowels, as well as the adhesions, fascia and subcutaneous tissue in subjects with and without adhesions. Comparatively, there was more variation in TGF-β than TGF-β3 expression in the absence of a relationship to age or gender. Furthermore, adhesions express a significantly higher TGF-β1 and had the highest TGF-β1:TGF-β3 ratio, with lowest levels and ratio detected in omentum, small and large bowels, in contrast the uterus expresses higher TGF-β3, with lowest levels detected in subcutaneous tissue and large bowels. In subjects with adhesions, the adhesions express significantly more TGF-β1 compared to intact parietal peritoneum. Since TGF-β is expressed differently in these tissues and tissue injury often alters the expression of TGF-β, we proposed that tissues with a higher basal expression of TGF-β are more predisposed to develop more adhesions compared to others (114).

5.2. The role of Cytokines

The interleukins are another key group of regulators of peritoneal wound healing and adhesion formation. IL-1 is considered a key proinflammtory cytokine and both IL-1α and IL-1β are expressed during the early stage of wound healing. However, these cytokines are also expressed by mesothelial cells and fibroblasts, and are detectable in peritoneal fluid, suggesting their influence on other phases of wound healing including tissue fibrosis (9,10,12,26,108,115,120,124). The peritoneal expression of IL-1 increases following surgical-induced injury, peaking during the first week and its inhibition was shown to reduce the incidence of postsurgical adhesion formation. In contrast to IL-1α or IL-β, M-CSF, G-CSF and IL-6 failed to trigger the proliferation of mesothelial cells, although they are expressed by these cells (124). GM-CSF also induced its own expression and the expression of TGF-β1 in peritoneal fibroblasts. TGF-β is a potent chemoattractant factor for macrophages and fibroblasts, whereas GM-CSF promotes macrophage uptake of apoptotic neutrophils (118). The interactions between TGF-β and GM-CSF in the peritoneal environment may act as an important regulator to maintain a balance between the inflammatory and immunosuppressive activities of GM-CSF and TGF-β, respectively. Furthermore, GM-CSF markedly increased the expression of TGF-α by macrophages, while IL-1β, IFN-γ, IL-6 and TNF-α induced the expression of PDGF (125). Analysis of IL-1α, IL-1β, TNF-α, PDGF, TGF-β1, and bFGF expression in scar biopsies led to the hypothesis that impaired production of cytokines such as IL-1α decreases, whereas augmented PDGF production increases matrix formation in hypertrophic scars (126).

Another cytokine, IL-2, is synthesized by activated T helper cells and is a mitogen for T, NK, and B cells. In addition, IL-2 stimulates IFN-γ, IL-3, IL-4, IL-5, and GM-CSF production. Mesothelial cells have not been shown to express IL-2, IL-3, IL-4, or IL-5 (124); however, a human mesothelial cell line expresses these cytokines, and they are detected in human peritoneal fluid (11,12,127) that locally can act to modulate the expression of GM-CSF and other cytokines. In addition, IL-2 has been shown to

increase the survival of mice with peritonitis (128). IL-4, a key Th2 cytokine, produced by T cells, mast cells, and basophiles, suppresses the development of Th1 cells. IL-4 has been found to stimulate connective tissue fibroblasts, accumulation of ECM, production of IL-6 and inhibit the expression of IL-1, IL-8, and TNF-α, it is considered a key wound healing cytokine by influencing the pathogenesis of fibrotic disorders and inflammatory response (129-131). Topical application of IL-4 in experimental wounds appears to enhance, whereas, IL-4 antisense oligonucleotides inhibit the rate of wound healing (132). IL-5 is major product of NK cells that regulates eosinophil differentiation, and increases B cell proliferation and T cell cytotoxicity; and combined production of IL-4 and IL-5 results in mast cell and eosinophil stimulation (129). IL-6 is produced by a variety of cells including mesothelial cells and fibroblasts, and induces IL-2 and IL-2 receptor expression, while it inhibits TNF-α production, providing a negative feedback for limiting the acute inflammatory response. The upregulation of IL-6 production associated with a variety of chronic inflammatory and autoimmune disorders, including peritonitis (11,26,124-129), implicate another member of the interleukins family in mediating peritoneal mesothelial cells activities. IL-6 is positively regulated by IL-1, TNF-α VEGF and PDGF, whereas it is inhibited by IL-4 and IL-10 (133-136). Because IL-6 has a stimulatory action of endothelial cell proliferation and VEGF expression, it may alter the outcome of angiogenesis that results in adhesion formation (135). IL-6-deficiency has been reported to result in a significant delay in wound healing, characterized by minimal epithelial bridge formation, decreased inflammation, and granulation tissue formation, which was reversed by a single dose of recombinant IL-6 or IP injection of an expression plasmid containing the full-length IL-6 cDNA (136). Treatment with rIL-6 also reconstituted wound healing in dexamethasone-treated immunosuppressed mice (136). Elevated expression of IL-6 in peritonitis and following surgically-induced peritoneal injury further implicates IL-6 in peritoneal adhesion formation (11,12).

IL-8 which belongs to the chemokines family, is the main chemotactic factor for neutrophils. It upregulates the expression of cell-surface adhesion molecules, such as endothelial leukocyte adhesion molecule and intracellular adhesion molecule, that enhance neutrophil adherence to endothelial cells and facilitates their migration through vessel walls. IL-8 is expressed in parietal peritoneum and adhesions, and its expression in mesothelial cells is regulated by IL-1 and TNF-α (11,12,137). Increased production of IL-8 in nonhealing wounds has been shown to prevent keratinocyte replication without effecting fibroblasts. However, IL-8 inhibits the rate fibroblast migration into the collagen lattice, a process reversed by co-treatment with indomethacin through the inhibition of prostaglandin production (138). Although elevated levels of IL-8 may delay wound healing by retarding wound closure (138), topical application of IL-8 on human skin grafts in a chimeric mouse model has been reported to enhance re-epithelialization, though it induced a significant reduction in wound contraction (139). These results further indicate that IL-8 can sequentially influence all phases of wound healing, including tissue remodeling associated with adhesion formation.

IL-10 is produced by Th2 cells and inhibits Th1 function by preventing the production ofTh1 cytokine, such as IL-4 and IFN-γ Thus, it is considered a T cell cross-regulatory factor and a key anti-inflammatory cytokine (26,140,141). IL-10 is expressed by a variety of cell types, including CD4+ and CD8+ T cells, and activated B cells, mesothelial cells and fibroblasts, and it is detectable in wounds throughout the healing period (142). IL-10 expression peaks immediately after injury and returns to normal levels within 24h, but is increased again at a later time point. IL-10 has been shown to inhibit overexpression of MCP-1, MIP-1α, IL-1β, IL-6, and TNF-α following tissue injury, and neutralizing antibody to IL-10 inhibited the infiltration of neutrophils and macrophages at the site of injury (142). IL-10 is also expressed during peritoneal wound healing. Although postoperative application of IL-10 did not alter the outcome of peritoneal adhesion formation, it was found to be effective at reducing the incidence of adhesion formation (143). Interestingly, peritoneal fluid contains low levels of IL-10 during the postoperative period that do not correlate with adhesion scores, suggesting that the endogenous IL-10 may not play a role mediating adhesion-free peritoneal healing (143). In contrast, IL-10 has been reported to disseminate bacterial outgrowth during peritonitis, and it protected mice from lethality by attenuating a systemic inflammatory response through a mechanism involving inhibition of TNF-α release (144). Mice deficient in IL-10 that were subjected to ischemia and reperfusion injury experienced a higher rate of mortality and more severe tissue injury, characterized by epithelial hemorrhagic necrosis, upregulation of adhesion molecules, neutrophil infiltration, as well as the production of TNF-α and IL- 6 (152,145). It has been suggested that IL-10 exerts an anti-inflammatory action during reperfusion injury, possibly by regulating the early stress-related genetic response, adhesion molecule expression, neutrophil recruitment, and subsequent cytokine and oxidant generation (52,145,146).

IL-10, as well as IL-4, inhibits IL-12 production, an important cell-mediated inflammatory cytokine that plays a key role in wound healing. IL-12 is produced by activated macrophages, and among its key biological activities is the activation and proliferation of T cells, and NK cells, NK cell cytotoxicity, and IFN-γ production (147). Immunoneutralization of IL-12 in septic peritonitis not only resulted in increased mortality, but also promoted a shift away from IL-12 and IFN-γ in favor of IL-10 production (148). IL-13 like IL-10 is a key anti-inflammatory cytokine, and is closely related to IL-4; it is expressed primarily by T cells, mast cells, and activated basophils. The anti-inflammatory activity of IL-13 is due in part to its ability to inhibit inflammatory cytokines such as IL-1ß, TNF-α, IL-8, and IL-6 and induce IL-1 receptor antagonist and IL-1 type II receptor expression (149-150). IL-15 is another novel cytokine that shares many of its biological functions with IL-2 and is expressed in various cell types including mesothelial cells. IL-15 is expressed as two isoforms due to alternative splicing, with a different pattern of expression

and regulation than that reported for IL-2 (151-152). IL-15 is a key regulator of the local innate tissue inflammatory response and adaptive immunity, in particular that associated with NK cell proliferation, cytotoxic killing, and IFN-γ and TNF-α production (151-153). IL-13 and IL-15 are both expressed in peritoneal serosal tissue and adhesions, and are present in peritoneal fluid (154). A comparative analysis of peritoneal fluid content of IL-13 and IL-15 in women with endometriosis, which causes peritoneal inflammation and adhesion formation, in women who developed peritoneal adhesions unrelated to endometriosis, and in women with normal pelvic anatomy showed that these two cytokines may play a key role in mediating abnormalities associated with these disorders including adhesion formation (154). Interestingly, IL-13 and IL-15 differentially regulate TNF-α and TNF-α receptors expression, and TNF-α receptor type I content in the peritoneal fluid of women with peritoneal adhesions differs from those with endometriosis. The results of these studies, as well as reports demonstrating a diverse pattern of expression and distinct biological actions for IL-13 and IL-15 in other cells and tissues, suggest that these cytokines play a key role in both normal biologic functions and in pathologic conditions affecting the peritoneal cavity.

TNF-α also induced the expression of IL-18, another key inflammatory cytokine related to IL-1, while EGF and TGF-α inhibited its expression without influencing IL-18 protein release (155). There was also an increase in IL-18 expression during the cutaneous wound repair that was closely correlated to infiltration of neutrophils known to produce TNF-α (156). A genetically diabetic db/db mouse, with a prolonged wound inflammatory phase, had elevated expression of IL-18 during the late phase of repair and an absence of IFN-γ, despite the presence of subsets of leukocytic cells at the wound site that are known to produce IFN-γ in response to IL-18. Interestingly, TGF-β expression at the wound site may have a counterregulatory action on IL-18-induced IFN-γ expression, as TGF-β suppressed the production of IFN-γ by peripheral blood mononuclear cells following IL-18 induction (157). IL-10 and IFN-γ interaction also resulted in upregulation of TNF-α expression (158), a key proinflammatory cytokine with functions similar to IL-1 and IL-6. Patients with peritonitis had elevated levels of TNF-α, IL-1α, IL-6 and their soluble receptors, as well as IFN-α and IL-10 in the peritoneal fluids (11,12).

5.3. The role of Chemokines

Chemokines, such as MCP-1, IL-8, GRO*a*, IP-10, and Mig, are sequentially and differentially expressed during the phase-specific infiltration of leukocyte subsets in human wound healing (159,160). The timing of MCP-1 and MIP-1 expression in the wound suggests that MCP-1 is critical during the early phase, while MIP-1 may regulate the events occurring during the later phase of the repair process (161). In addition, the induction of MCP-1 in keratinocytes following wounding is attenuated by IL-1α- and TNF-α and augmented by IL-8, a functional human homolog to murine MIP-2 (162). Interestingly, wound re-epithelialization and collagen synthesis in MIP-1α deficient mice were nearly identical to that occurred in wild-type, whereas MCP-1 deficiency resulted in a significant delay in these parameters without affecting the number of wound macrophages (163), suggesting that monocyte recruitment into wounds is independent of MCP-1. IP-10 is expressed in human dermal wounds, with highly elevated levels in a number of chronic inflammatory conditions, including psoriasis. Transgenic mice that constitutively express IP-10 in keratinocytes developed normally and did not spontaneously recruit leukocytes into the skin or other organs that expressed the transgene. However, these mice had an abnormal wound healing response characterized by a more intense inflammatory phase and a prolonged and disorganized granulation phase associated with impaired blood vessel formation (164). IP-10 also inhibits EGF- and HB-EGF-induced dermal fibroblast motility without affecting either the basal or EGF receptor-mediated mitogenesis (165). These results suggest that IP-10, by inhibiting neovascularization, may have a novel biologic activity as an inhibitor of wound healing (164). The expression of MGSA/GRO and their receptor, the type B IL-8 receptor (IL-8RB), is restricted to sites populated by differentiated keratinocytes in normal skin. However, wounds that resulted in hypertrophic scaring expressed IL-8RB only in specific locations within epidermal and dermal compartments of normal and hypertrophic epidermal wounds and in granulation tissues (166). In addition, fibroblasts prepared from keloids or normal skin fibroblasts do not express MGSA/GRO; however, its expression induced following treatment with IL-1 and inhibited by glucocorticoid (167). Although these chemokines are synthesized and released by activated peritoneal fluid resident cells i.e. macrophages, neutrophils, mast cells and T cells, little is known about their expression during the peritoneal wound repair process. Intact peritoneum and adhesions express MCP-1, RANTES and IP-10 (168). During peritoneal wound healing, MCP-1 expression is detected within 48 hrs of postinjury that remained elevated until the fourth postoperative day and daily IP administration of anti-MCP-1 antibody for 6 days significantly reduced the incidence of adhesion formation (169). In addition, the peritoneal fluid content of MCP-1 was elevated in women with adhesions; however, there was no incremental effect of MCP-1 level on adhesion formation in patients with endometriosis (170). However, peritoneal serosal tissues and adhesions as well as mesothelial cells and adhesion fibroblasts, also express MCP-1, IP-10 and RANTES, and may turn serve as potential sources of these chemokines in peritoneal fluid. Elevated expression of MCP-1, IP-10 and RANTES in surgically-induced adhesions in rats and mice was directly proportional to the incidence of adhesions (168,169).

6. TISSUE REMODELING IN ADHESION DEVELOPMENT

Extracellular matrix (ECM) deposition and tissue remodeling are critical to all phases of normal wound healing most notably cell migration, growth and differentiation, angiogenesis and tissue fibrosis (9,67,69,108,171). During wound healing, wound cells are in dynamic contact with ECM, and through the interactions and communication mediated by integrins, promote

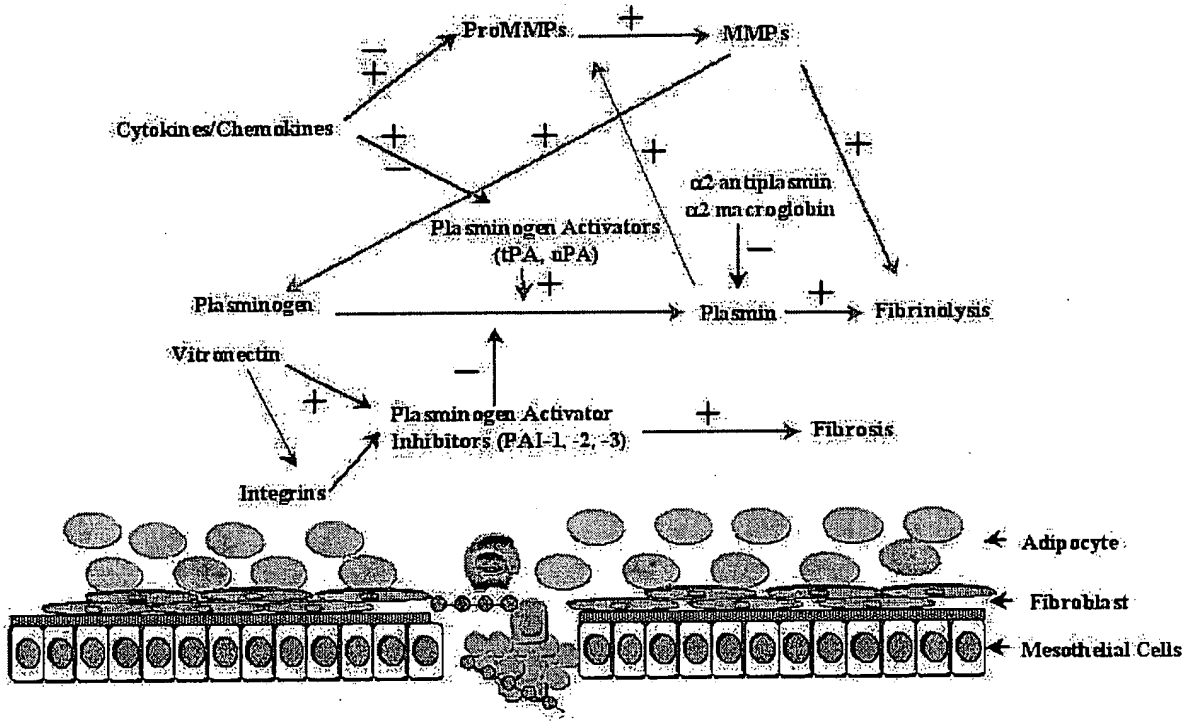


Figure 4. Schematic representation of fibrinolytic system and metrix metalloproteinases interactions, and their regulation by cytokines, chemokines and adhesion molecules that result in peritoneal wound healing and adhesion formation.

intracellular signals that regulate the above process (21,108,172). In addition, wound cells synthesize and release several proteolytic enzymes that hydrolyze various components of the ECM, such as collagens, fibronectin, vitronectin, laminin, elastin, and proteoglycans, allowing wound healing to proceed (108,171,172). Immediately after tissue injury, the exposure of fibrillar collagen to blood promotes aggregation and activation of platelets and the release of chemotactic factors, cytokines, chemokines, growth factors, proteases, etc., that regulate the ECM. Fibronectin, a major ECM component, is deposited in the wound as a part of preliminary matrix acting as a scaffold for cell migration and collagen deposition, and later regulation of re-epithlialization and wound contraction (9,108,171-176). Proteolytic degradation of collagens and fibronectin results in formation of smaller fragments that attract inflammatory cells, and later fibroblasts, into the injured area. Fibronectin fragments, but not intact fibronectin, also induce the expression of MMPs and PA, and increase proteoglycan content in a concentration-dependent manner (177,178). These fragments also enhance the release of several growth factors and cytokines that bind either to their specific cell surfaces or to the ECM following their release from producer cells (177,178). Many of these cytokines regulate proteases and ECM expression. Neutralization of the action of these cytokines reduced fibronectin fragment-mediated MMP-3 release and suppressed proteoglycan synthesis (178). As illustrated the interactions among components of the ECM, proteases and cytokines are regulated through a feedback mechanisms that are critical to the inflammatory response, angiogenesis and tissue repair occurring during peritonitis and peritoneal wound healing processes (Figure 4).

Although a very limited information is available regarding the expression and role of ECM in peritoneal environment during wound healing and adhesion development, collagen type I, type III and fibronectin are localized in the peritoneal wall and their expression is detected in peritoneal mesothelial cells and adhesion fibroblasts (101,109,179). TGF-β in a cell specific manner increases the expression of fibronectin and procollagen I in human adhesion fibroblasts, whereas it induces collagen type III, with a limited effect on collagen I in mesothelial cells (179,180). Because overproduction of TGF-β is associated with increased incidence of adhesion formation, modulation of the expression of ECM in adhesion fibroblasts may partly account for TGF-β induced adhesions (179,180). Peritoneal mesothelial cells and the serosal surface of several peritoneal organs and parietal peritoneum also express integrins, such as αv and β3 (99,101). These integrins specifically bind fibronectin and vitronectin expressed by peritoneal serosal tissue and mesothelial cells, and are regulated by TGF-β (79,107). Vitronectin deficiency has been reported to cause only a slight delay in dermal wound healing and was associated with a temporal increase in uPA and tPA activity in microvessels and decreased angiogenesis in response to tissue injury (180). The interaction of vitronectin with PAI-1, integrins and MMPs, factors having individual and interactive biological activities critical to the outcome of wound healing, further imply the

importance of vitronectin in peritoneal wound healing and adhesion formation.

ECM components are also important in regulating the expression of growth factors and cytokines in an interactive manner (175,176). In vitro experiments have shown that the expression of TNF-α and PDGF are dependent on the existence of ECM proteins (176,181). In contrast, the expression of TGF-β is constitutive, and IL-1, which is stimulated by bacterial endotoxin, is ECM-independent (182). The importance of ECM becomes further apparent when considering the association between numerous cytokine and growth factor soluble receptors with the ECM compartment (183-185). In some cases these specific ligand-receptor interactions involve either a single subunit or a complex of two or more subunits that are commonly shared among certain cytokines to initiate a cascade of intracellular signal transduction. However, many of these receptor molecules, often representing the ligand binding domain of the receptor complex, are detectable in naturally-occurring soluble forms in the serum, plasma, urine and in various cell culture-conditioned media. These include soluble receptors for interleukins, G-CSF, M-CSF, GM-CSF, TNF-α, EGF, TGF-α, TGF-β and PDGF, as well as IGFBPs. For instance, the protoglycan, decorin, as well as other ECM components such as biglycan and fibromodulin, associate and result in inactivation of TGF-β (22,103-105). TGF-β can downregulate the expression of decorin, while upregulating biglycan and inhibiting fibrilogenesis. Although the in vivo functions of these soluble receptors are unknown, potentially they can restrict the biological activities of their receptive ligands (antagonists), act as scavengers, control the physiological levels of these cytokines, or function as transport proteins to be presented to cell surface receptors.

Tissue remodeling and the induction of fibrosis occur not only by promoting chemotactic recruitment of fibroblasts and increasing in the deposition of ECM proteins, but also through the differential regulation of proteolytic enzymes that degrade the ECM (9,11,67,108). The earliest event in the wound repair process involves deposition of fibrin-rich exudates, referred to as provisional matrix (9,108). This matrix contains a variety of substances that are key elements in the initiation of wound healing; however, the resolution of this matrix is also necessary for the continuation of the healing process (9,108). The components of the fibrinolytic system and MMPs interact in a variety of ways to regulate many of the processes that are required for ECM proteolysis (16,17,108).

6.1. The role of Fibrinolytic System

Plasmin, a broad-range serine protease, cleaves a number of ECM components and degrades fibrin and its proenzymes, tPA and u-PA, are inhibited by PAIs, in particular, PAI-1 (16,17). Different cell types, including wound cells, express components of the fibrinolytic system (9,11,12,16,17). In the peritoneal cavity, peritoneal macrophages produce tPA, PAI-1, and their receptors, but the major contributor to fibrinolytic activity in the abdomen appears to be the mesothelial cell (11,12,186). Mesothelial cells mainly express the activators of fibrinolysis, whereas its inhibitors are widely distributed in a variety of tissues (11,12,187). This suggests that an intact mesothelium seems to be crucial in maintaining the balance of fibrin deposition and degradation. Gene targeting has provided important evidence supporting the role of fibrinolytic components in various physiological and pathological events including wound healing. Studies of individual and combined deficiencies of uPAR and tPA have shown that a deficiency in uPAR/tPA does not induce as profound an impairment in wound repair as that seen with uPA/tPA deficiency following full-thickness skin injury (188). These studies suggest that uPA alone is sufficient to clear fibrin deposits and support wound healing without the benefit of either uPAR or tPA. However, plasminogen deficiency causes severe thrombosis with delayed wound healing, in part due to impaired keratinocyte migration. Deficiency in both plasminogen and fibrinogen revealed that removal of fibrinogen from the extracellular environment alleviates the impairment associated with plasminogen deficiency and corrects healing, implying a fundamental physiological role for plasminogen in fibrinolysis (189,190). Furthermore, in vitro studies strongly support the importance of plasmin in angiogenesis; however, angiogenesis and fibrinolytic activity do not always appear to correlate with in vivo observations as suggested by the evidence of normal angiogenic activity during wound healing in the presence of plasminogen-deficiency (189,190). Deficiency in vitronectin was also associated with an increase in uPA and tPA activity (180). A further role of fibrinolysis in wound healing is suggested by the action of D dimer, a plasmin proteolytic fragment of fibrin, on the induction of PAI-1 expression, constituting a negative feedback mechanism in which specific fibrin fragments control the persistence fibrin at sites of inflammation and fibrosis (191). In addition, the annexin II heterotetramer (AIIt) that binds tPA, plasminogen, and plasmin stimulates the tPA-dependent conversion of plasminogen to plasmin and AIIt is the key physiological receptor for plasminogen on the extracellular surface of endothelial cells (192)(Figure 4).

Tissue trauma, ischemia and infection, events that increase the incidence of adhesion formation, are associated with reduced peritoneal fibrinolytic activity due to the rapid reduction in tPA activity and the increase in PAI-1 production that occurs shortly after post-operation (11,193). It seems possible that reduced tPA activity, in conjunction with over-expression of PAI-1 and quenching of t-PA, may explain the high frequency of adhesion reformation after adhesiolysis (1,11). A lower tPA to PAI-1 ratio was also observed immediately after surgery in patients who underwent hepatic versus colorectal resection. Extensive hypercoagulation and hypofibrinolytic activation during the late postoperative period also impair wound healing (9,11,194,195). Because impairment of peritoneal fibrinolysis following tissue injury leads to the development of adhesions, the effectiveness of intraperitoneal applications of recombinant tPA has been tested for the prevention of adhesions (194-198). Intraoperative IP administration of tPA has been shown to alter the strength, and extent of fibrous bands and wound strength with significantly lower plasma PAI-1 and PAI-2 production one to two weeks postsurgery. These findings

suggest that tPA can reduce the incidence of postoperative peritoneal adhesions without impairing wound healing. The effect of rtPA in overcoming the inhibition of fibrinolysis was dose related, as low levels showed no effect on adhesion formation, while higher concentrations reduced adhesions that paralleled wound hydroxyproline content (198). However, as indicated in many other studies, the levels of rtPA required to alter or prevent peritoneal adhesion formation not only impair the early phase of wound healing, but can also be detrimental (199). Intraperitoneal administration of anti-PAI-1 antibodies has also been reported to reduce the incidence of adhesion formation in surgically-induced peritoneal injury, possibly by limiting the availability of PAI-1 to inhibit tPA (200).

6.2. The role of Matrix Metalloproteinases

In addition to the fibrinolytic system, MMPs, a family of zinc-dependent endopeptidases, as a group can degrade essentially all components of the ECM that control tissue remodeling of newly formed granulation tissue. So far, 28 members of the MMP family have been identified. Based on their structure and substrate specificity, MMPs are divided into subgroups that include: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11), matrilysin (MMP-9), and the membrane-type MMPs (MT-MMP) such as MT-MMP-1, etc (9,67,69,108,201). The catalytic activity of MMPs is controlled, at least in part, by their physiological inhibitors, TIMPs, which are comprised of TIMP-1 –2, -3 and -4 (67-70,202). MMPs typically are not constitutively expressed; however, they are induced in tissues that normally undergo extensive remodeling, such as wound tissue, and in response to various inflammatory conditions. In addition, MMPs are regulated by cytokines, growth factors, hormones, and cell-cell and cell-matrix interactions (67-70,201,202). In contrast, the expression of TIMPs is widespread in many tissues and is regulated in coordination with MMPs expression (67-70). TIMPs have growth factor-like activity in various cell types, alter angiogenesis and are associated with several other cellular activities (70,202). MMPs are produced as inactive proenzymes and require activation, achieved by various factors including serine proteases, such as plasmin, trypsin and neutrophil elastase (67-70,201,202). The temporal expression profiles for MMP-1, MMP-2, MMP-7, MMP-9, MT1-MMP, as well as TIMP-1, TIMP-2, and TIMP-3 observed during the inflammatory, granulation, and early tissue remodeling phases of excisional skin repair indicated that their induction and peak expression coincided with the well-characterized inflammatory and granulation phases (203). The expression of MMP-2, MMP-14, and TIMP-1 mRNAs was increased in inflamed areas in ulcerated colonic mucosa, with substantial increases in MMP-1 and MMP-3 expression that correlated with the histological degree of acute inflammation (204). Studies of the temporal activity of MMP-2, MMP-3, MMP-7, and MMP-9 during normal dermal repair also revealed that MMP-2 is a major gelatinolytic MMP (205) that participates in physiological turnover of ECM, whereas MMP-9 is important in the early phase of ulceration and in the healing process (206). Furthermore, the cellular distribution of MMPs suggests that these enzymes may contribute to anastomotic dehiscence, but only in the immediate postoperative period (207).

TIMP-1 is not expressed in the epidermis, but is co-localized with MMP-9, and MMP-3 during the hyperproliferative phase at the mesenchymal/epidermal border of granulation tissue. At the later phases of wound healing there is an increase in MMP-8 and MT1-MMP expression, and TIMPs are colocalized with MMPs in granulation tissue. At the completion of re-epithelialization, the expression of MMPs and TIMP-1 in epidermal and dermal compartments declined to near-basal levels, whereas the macrophage-specific metalloelastase (MMP-12) reached maximum expression. Systemic glucocorticoid treatment, which results in impaired wound healing, led to a nearly complete shut-off of MMP-12 expression (67). Infected and chronic wounds have been associated with persistent elevation of MMP-9, and its detection in postoperative wound fluids is considered to be an early indicator of impaired healing and relates to the amount of collagen deposition later in the wound healing process (208). Other studies have shown that MMP-2 is localized to the connective tissue fibroblasts and endothelial cells during all phases of wound healing. In contrast, mucosal epithelium is practically devoid of MMP-2, and only the basal cell layer expresses MMP-9 in the migrating epithelial cells, with strong expression in granulation tissue. Cytokines such as TGF-β1, IL-1β, bFGF, TNF-α, and IFN-γ differentially regulate the expression of MMPs and TIMPs in various cells types; IL1-β and TNF-α enhance MMP expression (67-70,201,202). Human dermal wound healing has also been shown to be associated with age-related increases in MMP-2 and MMP-9, MMP-2 was found in association with epidermal structures, while MMP-9 was observed in inflammatory cells (210). MMP-8 and MMP-13 were clearly reduced in the skin wound extracts of chemically modified tetracycline (CMT-8) treated rats compared to ovraictomized rats, suggesting that CMT-8 and estrogen have a beneficial effect on skin wound healing, possibly by increasing the collagen content and by reducing MMP-mediated collagenolysis (211).

We have provided evidence that peritoneum and adhesion tissues express MMPs and TIMPs with TIMP-1 levels significantly higher in fibrous adhesions than in the peritoneal serosal tissue (76,77). This increase in TIMP-1 expression in fibrous adhesions paralleled the expression of TGF-β1 and integrin (99,114). Under in vitro conditions, TGF-β1 inhibits the expression of MMPs and increases TIMPs, decreasing matrix degradation and increasing tissue fibrosis (22,67). Studies using isolated peritoneal mesothelial cells and adhesion fibroblasts indicated that TGF-β1 also increases the level of collagens, fibronectin and TIMP-1, while it reduces the expression of MMP-1 mRNA by these cells (75,107,109,179). Mesothelial cells also express MMP-1, MMP-3, TIMP-1 and TIMP-2 mRNA and protein at various levels, with the expression TIMPs being quantitatively the highest (75,107,109,179). Treatment with TGF-β1 resulted in a significant increase in the expression of TIMP-1, but not TIMP-2, mRNA in mesothelial cells, while down-regulating MMP-1 and MMP-3 (75). In contrast, TGF-β1

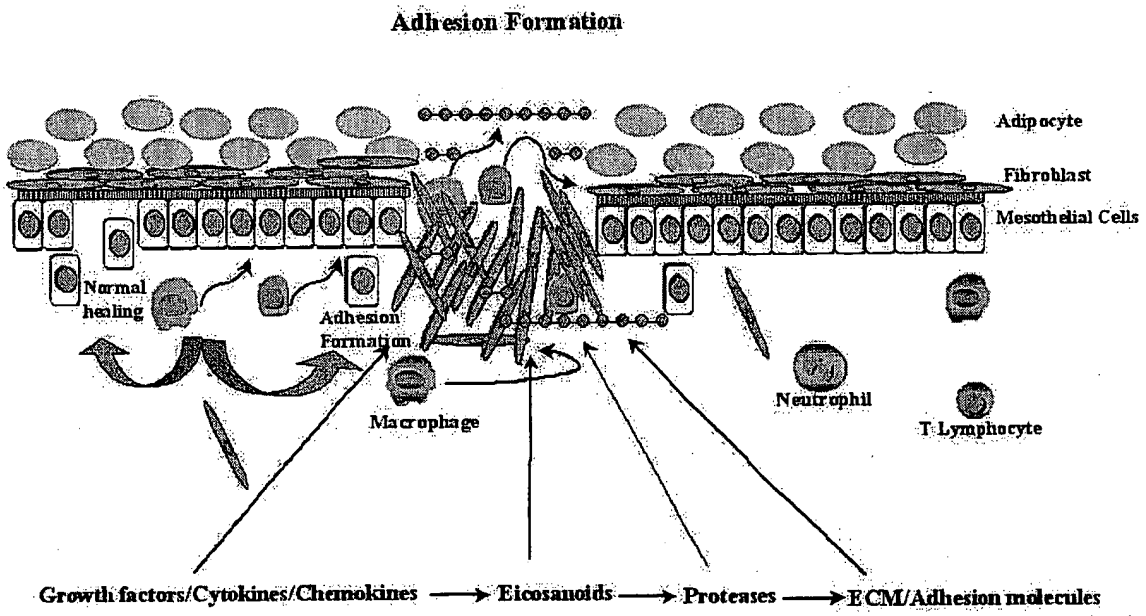


Figure 5. Schematic representation of events, and cytokines, growth factors, eicosanoids, proteases, adhesion molecules that regulate the excess migration and proliferation of fibroblasts that leads to peritoneal adhesion formation.

increased the release of MMP-1 and TIMP-1 by mesothelial cells, but majority of MMP-1 was complexed with TIMP-1 (75). These data provide further evidence that proteolytic enzymes, whose expression is partly regulated by TGF-β and other cytokines, may influence ECM turnover and the incidence of adhesion formation (Figures 4 & 5).

The blocking of MMP activity has been studied for its potential therapeutic efficacy in controlling pathologic processes. Synthetic MMP inhibitors, most notably the hydroxymates, have been engineered for this purpose and are presently undergoing clinical trials. These inhibitors may have broad or specific MMP inhibitory activity. However, non-matrix degrading capabilities of MMP have also been recognized, and include cytokine activation, processing of proteins to molecules of distinct biologic function. Thus it is less clear whether nonselective inhibition of MMP activity for all pathologic processes involving MMP is appropriate (67,212). Treatment of wounds with tetracycline analogues that inhibit MMP-2, but not MMP-9 production, is reported to inhibit migration and growth of mucosal and skin keratinocytes and keratinocyte growth. TGF-β treatment increased keratinocyte migration as well as the cell-associated and secreted MMP-2 production with partial conversion into an active form. Batimastat totally blocked TGFβ-induced keratinocyte migration (212). Another MMPs inhibitor, GM 6001 topically applied to the dermal wound did not influence the degree of dermal inflammatory cell infiltrate or epithelial proliferation, although it reduced re-epithelialization which was not due to interference with the inflammatory response or epithelial proliferation (212). In a comparative study of GM-6001 action in dermal and peritoneal wound healing and peritoneal adhesion formation, it has been found that MMP inhibitor has limited effect on the outcome of peritoneal healing and adhesion formation (213). In colonic anastomosis treatment with BB-1101, a synthetic broad-spectrum MMP inhibitor, increased anastomotic breaking strength without affecting collagen accumulation or infiltration of neutrophils in anastomotic area. The result indicates that alteration in MMPs expression during the critical early postoperative phase may increase the risk of anastomotic dehiscence (214).

As illustrated these proteases and their inhibitors are key regulators of many phases of wound healing. Since they are expressed differently in peritoneal wounds and adhesion tissues, and tissue injury is often associated with altered expression of proteases, we proposed that tissues with a higher basal expression of these molecules are more predisposed to develop more adhesions compared to others.

7. PROSPECTIVE

Whether induced by infection, inflammation, ischaemia, and/or surgical injury, peritoneal adhesions are the leading cause of pelvic pain, bowel obstruction and infertility, and cause a substantial burden during reoperative procedures as well as increasing medical cost. It is also clear that while postsurgical peritoneal wounds heal without adhesions in some patients, others develop severe scarring from seemingly equal procedures; in addition in the same patient, adhesions can develop at one surgical site and not in another. The mechanisms underlying the predisposition to form adhesions as well as their site specificity are completely unknown. However, a large number of intraperitoneal surgical procedures are performed each day in the USA, and thus many patients are at risk of developing postoperative adhesions. Therefore, the understanding of adhesion formation at the molecular level is essential and in the absence of such information,

attempts to prevent patients from developing adhesions will remain an empirical process.

The unprecedented advancement in molecular biology during the past decade has led to the identification of many biologically active molecules with the potential of regulating inflammatory and immune responses, angiogenesis and tissue remodeling, events that are central to normal wound healing as well as to tissue fibrosis associated with adhesion formation. The list of molecules that modify the wound healing process has also grown substantially, with increasing insight into their importance in the development of tissue fibrosis. However, their major roles in peritoneal biological functions and the adhesion formation remain speculative at best. Interestingly, there are common and overlapping biological functions among many growth factors, cytokines, chemokines and proteases that are evolved from the recruitment of multiple signaling molecules with similar downstream pathways. These molecules are also able to compensate for the function of a deleted gene product by using alternative pathways to trigger the full-scale activation of cellular response. Such functional pleiotropy and redundancy, a characteristic feature of many of these molecules, has been attributed to the molecular structure of their receptor and to the binding protein system. Elucidating how key regulatory molecules of the signaling pathways cooperate and interact, as well as factors involved in their downstream cascades, in peritoneal mesothelial cells and adhesion fibroblasts may allow the identification of molecules that are potentially vital in peritoneal repair, cellular invasion, and tissue fibrosis. This information could allow design and exploration of the use of specific modulators (inhibitor/stimulator) for their potential therapeutic applications in adhesion prevention and other disorders such as endometriosis and peritoneal cancers. For instance, several therapeutic interventions have been shown to be effective in modulating cellular behavior in disorders that could be potentially useful in modulating peritoneal wound healing and adhesion formation. These include synthetic inhibitors of cell invasion (marimastat, Neovastat, AG-3340), adhesion (Vitaxin), or proliferation (TNP-470, thalidomide, Combretastatin A-4). Compounds that interfere with angiogenic growth factors (IFN-α, suramin, and analogues) or their receptors (SU6668, SU5416), as well as endogenous inhibitors of angiogenesis (endostatin, IL-12), are currently being evaluated in clinical trials for the treatment of a variety of disorders (215). Modification of cellular function through gene targeting is also a promising therapeutic modality. The incorporation of genetically modified peritoneal mesothelial cells might be useful in preserving the normal physiological function of peritoneum during peritoneal dialysis, and through the production of proteins with therapeutic property could safeguard the peritoneal membrane against injury. Such local genetic modification could also be applied to alter the behavior of a variety of pathological conditions affecting the peritoneum, including carcinomas, such as ovarian cancers mesotheliomas, and endometriosis. For instance, IP administration of antisense oligonucleotides, plasmids, and viral vectors that are been evaluated for their efficacy in cancer biology have a potential application in adhesion prevention. IP administration of Semliki Forest virus (SFV) particles encoding recombinant murine GM-CSF to alter recruitment and activation of inflammatory cells and ovarian tumor growth, revealed a high level of incorporation in peritoneal mesothelial cells (216). Due to the exclusive uptake of viral particles by mesothelial cells, ex vivo gene transfer into mesothelial cells isolated from the peritoneal membrane, genetically modified in vitro and subsequently re-implanted back onto the peritoneal cavity of syngeneic recipients for in vivo gene therapy. This use of genetically modified peritoneal mesothelial cells may be of therapeutic value in maintaining the fibrinolytic balance in the peritoneal cavity, altering peritoneal inflammation, angiogenesis, cell migration, and the development of peritoneal adhesions or peritoneal physiology to prevent membrane damage and to maintain dialyzing performance (217,218). Another therapeutic approach has been the IP delivery of recombinant therapeutic proteins from a universal microencapsulated cell line as an alternate method for gene therapy. This approach has proved effective for the treatment of several murine models of human genetic diseases. However, in scaling up to large animal models, IP implantations of microcapsules have been associated with an excessive inflammatory response and rapid degradation, although implantation at a surgical site induced less inflammation, and permitted longer-term survival of microcapsules. However, adhesions consist mainly of migrating fibroblasts and it appears that cell type-specific gene delivery is essential for in vivo gene therapy. Interestingly, retroviral vector particles derived from the spleen necrosis virus, which display the antigen-binding site of an antibody on the viral surface have been used to infect cells expressing a receptor recognized by such as antibody, an approach that can be applied using mesothelial cells (219). Mesothelial cells are easy to obtain and propagate from individual patients, and following their genetic manipulation to express therapeutic proteins at useful levels such cells appear to provide a tool with clinical potential based on ex vivo gene therapy (220).

In recent years several biodegradable devices have been either marketed or are in clinical trials to prevent postoperative adhesion formation (Table 1). Although their addition to the field of adhesion preventative devices is welcomed, they appear to have either a limited or insignificant usefulness in the prevention of adhesion formation. In addition, the choice and utilization of these devices most often has been determined by their availability to the investigator, rather than on the basis of rigorous scientific investigation. Because these devices are of potential use for site-specific delivery of bioactive molecules, a clear understanding of their biocompatablity with the peritoneal environment as well the most effective forms (membrane, gel or liquid) to carry and appropriately release these molecules is required.

8. ACKNOWLEDGMENTS

I would like to thank Drs. Joyce M. Koenig, R. Stan Williams and Barbara Bennett for their helpful suggestions, and many of my postdoctoral fellows and students for their research contributions in the field of peritoneal wound healing that made it possible to write this article.

Table 1. Current Products and Devices to Reduce Adhesion Formation

Product & Company	Composition	Indications
Interceed J&J/Eithicon)	Cellulose	Gynecological surgery
Preclude (W. L. Gore)	PTFE	Peritoneal reconstruction
Seprafilm (Genzyme)	Hyaluronic acid (Genzyme)	Gynecological & General surgery
Hydrogel (Biomatrix)	" " " " " " " "	Preclinical
Lubricoat (Lifecore Biomedical)	" " " " " " " "	IDE trials
Incert (Anika)	" " " " " " " "	Preclinical (de nova adhesions)
Adcon P (Gliatech)	Polysaccharide	General surgery
Atrigel (Atrix)	Polylactic acid-based polymers	Preclinical (denova adhesion)
Repel (Life Medical)	" " " " " " " "	IDE trail (Gyn/abd/pelvic surgeries)
Focal gel (Focal)	" " " " " " " "	Gynecological surgery
Atrigel (Atrix)		Preclinical
Flogel (Alliance Pharmaceutical)	Pluronics	IDE trials (Gyn & General surgeries)
BioElastics (Bioelastics Res. Ltd)	Elastin peptides	Preclinical
Sepragel (Genzyme)	HA	IDE (Gyn & General surgery)
Sepracoat (Genzyme)	" " " " " " "	Preclinical
Intergel (Lifecore)	" " " " " " "	Preclinical

9. REFERENCES

1. M.P. Diamond. & L.B. Schwartz: "Prevention of Adhesion Development". In: Endoscopic Surgery for Gynecologists. Eds: Sutton C and Diamond M.P, 2nd Edition, W.B. Saunders, London, England, 398-406 (1994)

2. Ellis H., B.J. Moran, J.N. Thompson, M.C. Parker, M.S. Wilson , D. Menzies, A. McGuire, A.M. Lower, R. J. S. Hawthorn, F. O'Brien, S. Buchan, & A.M Crowe. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. *Lancet* 353, 1476-1480 (1999)

3. Lower A.M, R.J. Hawthorn, H. Ellis, F. O'Brien, S. Buchan, A.M. Crowe. The impact of adhesions on hospital readmissions over ten years after 8849 open gynecological operations: an assessment from the surgical and clinical adhesions research study. *Brit J Obstet Gynecol* 107, 855-862 (2000)

4. Diamond M.P., & M.L. Freeman. Clinical implications of postsurgical adhesions. *Hum Reprod Update*.7,567-576 (2001)

5. Franklin RR, M.P. Diamond, LR. Malinak, B. Larsson, R.P.S. Jansen, S. Rosenberg, & B.W. Webster. Reduction of ovarian adhesions by the use of interceed. *Obstet Gynecol* 86, 335-340 (1995)

6. Diamond M.P and the Seprafilm Adhesion Study Group: Reduction of adhesions after uterine myomectomy by Seprafilm (HAL-F) A blinded prospective, randomized, multicenter clinical study. *Fertil Steril* 66, 904-910 (1996)

7. Diamond M.P. and the Sepracoat Adhesion Study Group. Reduction of De Novo Postsurgical Adhesions by Intraoperative Precoating with Sepracoat (HAL-C) Solution, A Prospective, Randomized, Blinded, Placebo-Controlled Multicenter Study. *Fertil Steril* 69, 1067-1073 (1998)

8. Becker JM, M.T. Dayton, V.W. Fazio, D.E. Beck, S.J. Stryker, S.D. Wexner, B.G. Wolff, P.L. Roberts, L.E. Smith, S.A. Sweeney, & M. Moore. Prevention of postoperative abdominal adhesions by a sodium hyaluronate-based bioresorbable membrane: a prospective randomized, double-blind multicenter study. *J Am Coll Surg* 183, 297-306 (1996)

9. R.A.F Clark. Wound repair. Overview and general consideration. In: The molecular biology of wound repair. Ed: Clark R.A.F. 2nd ed. New York: Plenum Press, 3-50 (1996)

10. Raftery AT. Regeneration of peritoneum: a fibrinolytic study. *J Anat* 129,659-64 (1979)

11. Topley N, R.K. Mackenzie, & J.D. Williams. Macrophages and mesothelial cells in bacterial peritonitis. *Immunobiology* 95,63-573 (1996)

12. Holmdahl L, & L.M. Ivarsson. The role of cytokines, coagulation and fibrinolysis in peritoneal tissue repair. *Eur. J Surg*, 165, 1012-9 (1999)

13. Muller S.A. K.H. Treutner, L. Tietze, M Anurov, S. Titkova, M. Polivoda, A.P. Oettinger, & V. Schumpelick. Efficacy of adhesion prevention and impact on wound healing of intraperitoneal phospholipids. *J Surg Res* 96, 68-74 (2001)

14. Furie B, & B.C. Furie. The molecular basis of blood coagulation. *Cell* 53, 505-518 (1998)

15. Gaffney P.J.Fibrin degradation products. A review of structures found in vitro and in vivo. *Ann N Y Acad Sci.* 936, 594-610 (2001)

16. Lijnen H.R. Elements of the fibrinolytic system. *Ann N Y Acad Sci* 936, 226-236 (2001)

17. Herrick S, O. Blanc-Brude, A. Gray, & G Laurent. Fibrinogen. *Int J Biochem Cell Biol* 31, 741-746 (1999)

18. Ruggeri Z.M. von Willebrand factor and fibrinogen. *Curr Opin Cell Biol* 5,898-906 (1993)

19. Bennett J.S. Platelet-fibrinogen interactions. *Ann N Y Acad Sci.* 936, 340-354 (2001)

20. Clemetson K.J, & J.M Clemetson. Platelet collagen receptors. *Thromb Haemost* 86, 189-197 (2001)

21. Holly S.P, M.K. Larson, & L.V. Parise. Multiple roles of integrins in cell motility. *Exp Cell Res* 261, 69-74 (2000)

22. Blobe G.C, W.P. Schiemann, & H.F. Lodish. Role of transforming growth factor beta in human disease. *N Engl J Med* 342, 1350-1358 (2000)

23. Heldin C.H, & B. Westermark. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 79, 1283-1316 (1999)

24. Iida N, M. Haisa, A. Igarashi, D. Pencev, & GR Grotendorst. Leukocyte-derived growth factor links the

PDGF and CXC chemokine families of peptides. *FASEB J.* 1996; 10: 1336-1345 (1996)
25. Jones G.E. Cellular signaling in macrophage migration and chemotaxis. *J Leukoc Biol* 68, 593-602 (2000)
26. Lowry SF. Cytokine mediators of immunity and inflammation. *Arch Surg* 128, 1235-1241 (1993)
27. Iwamoto R, & E. Mekada. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev* 11, 335-44 (2000)
28. Fitzpatrick F.A, & R. Soberman. Regulated formation of eicosanoids. *J Clin Invest* 107, 1347-1351 (2001)
29. Peplow P.V. Actions of cytokines in relation to arachidonic acid metabolism and eicosanoid production. *Prostaglandins Leukot Essent Fatty Acids* 54,303-317 (1996)
30. Sherman M, B. Weber, R. R. Datta & D.W. Kufe. Transcriptional and posttranscriptional regulation of macrophage-specific colony stimulating factor gene expression by tumor necrosis factor. Involvement of arachidonic acid metabolites. *J Clin Invest* 85, 442-447 (1990)
31. Gagnon L, L. Filion, C. Dubois, & M. Rola-Pleszczynski. Leukotrienes and macrophage activation: augmented cytotoxic activity and enhanced interleukin 1, tumor necrosis factor, and hydrogen peroxide production. *Agent Action* 26, 142-147 (1989)
32. Hori T, Y. Yamanaka, M. Hayakawa, S. Shibamoto, M. Tsujimoto, N. Oku, & F Ito. Prostaglandin antagonize fibroblast proliferation stimulated by tumor necrosis factor. *Biochem Biophys Res Commun* 174,758-766 (1991)
33. Chegini N, M.J. Rossi & L.E. Holmdahl. Cellular distribution of 5-lipoxygenase and leukotriene receptors in postsurgical peritoneal wound repair. *Wound Repair Reg* 5, 235-242 (1997)
34. Chegini N, & H. Rong. Postoperative exposure to glove powders modulates production of peritoneal eicosanoids during peritoneal wound healing. *Eur J Surg* 165, 698-704 (1999)
35. Muzii L, Marana R, Brunetti L, Margutti F, Vacca M, Mancuso S. Postoperative adhesion prevention with low-dose aspirin: effect through the selective inhibition of thromboxane production. *Hum Reprod* 13, 1486-1489 (1998)
36. Douma C.E, D.R. de Waart, D. Zemel, D.G. Struijk & R.T Krediet. Prostaglandin inhibition by intraperitoneal indomethacin has no effect on peritoneal permeability during stable CAPD. *Nephrol Dial Transplant* 16, 803-808 (2001)
37. Peng H, A.K. Cheung, L.G. Reimer, C.D. Kamerath & J.K. Leypoldt. Effect of indomethacin on peritoneal protein loss in a rabbit model of peritonitis. *Kidney Int* 59, 44-51 (2001)
38. Tager A.M, J.H. Dufour, K. Goodarzi, S.D. Bercury, U.H. von Andrian, & A.D. Luster. BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis. *J Exp Med* 192, 439-446 (2000)
39. Haribabu B, M.W. Verghese, D.A. Steeber, D.D. Sellars, C.B. Bock & R. Snyderman. Targeted disruption of the leukotriene B(4) receptor in mice reveals its role in inflammation and platelet-activating factor-induced anaphylaxis. *J Exp Med* 192, 433-438 (2000)
40. Gerard C, & B.J. Rollins. Chemokines and disease. Nat Immunol 2, 108-115 (2001)
41. Horuk R. Chemokine receptors. *Cytokine Growth Factor Rev* 12, 313-335 (2001)
42. Smiley S.T., J.A. King & W.W. Hancock. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167, 2887-2894 (2001)
43. Matsukawa A, C.M. Hogaboam, N.W. Lukacs, P.M. Lincoln, R.M. Strieter, & S.L. Kunkel. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *J Immunol* 163,6148-6154 (1999)
44. Fine JS, Jackson JV, Rojas-Triana A, Bober LA. Evaluation of chemokine- and phlogistin-mediated leukocyte chemotaxis using an in vivo sponge model. *Inflammation* 24, 331-346 (2000)
45. Call D.R, J.A. Nemzek, S.J. Ebong, G.B. Bolgos, D.E. Newcomb, G.K. Wollenberg, & D.G. Remick. Differential local and systemic regulation of the murine chemokines KC and MIP2. *Shock*.15, 278-284 (2001)
46. Kopydlowski K.M, C.A. Salkowski, M.J. Cody, N. van-Rooijen, J. Major, T.A. Hamilton & S.N. Vogel. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol*.163, 1537-1544 (1999)
47. Foussat A, K. Balabanian, A. Amara, L. Bouchet-Delbos, I. Durand-Gasselin, F. Baleux, J. Couderc, P. Galanaud, & D Emilie. Production of stromal cell-derived factor 1 by mesothelial cells and effects of this chemokine on peritoneal B lymphocytes. *Eur J Immunol* 31, 350-359 (2001)
48. Poznansky M.C, I.T. Olszak, R. Foxall, R.H. Evans, A.D. Luster, & D.T. Scadden. Active movement of T cells away from a chemokines. *Nat Med* 6, 543-548 (2000)
49. Steinhauser M.L, C.M. Hogaboam, A. Matsukawa, N.W. Lukacs, R.M. Strieter, & S.L. Kunkel. Chemokine C10 promotes disease resolution and survival in an experimental model of bacterial sepsis. *Infect Immun* 68, 6108-6114 (2000)
50. Orlofsky A, Y. Wu &M.B. Prystowsky. Divergent regulation of the murine CC chemokine C10 by Th(1) and Th(2) cytokines. *Cytokine* 12, 220-228 (2000)
51. Matsukawa A., C.M. Hogaboam, N.W. Lukacs, P.M. Lincoln, H.L. Evanoff, R.M. Strieter, & S.L. Kunkel. Expression and contribution of endogenous IL-13 in an experimental model of sepsis. *J Immunol* 164, 2738-2744 (2000)
52. Witowski J., A. Thiel, R. Dechend, K. Dunkel, N. Fouquet, T.O. Bender, J.M. Langrehr, G.M. Gahl, U. Frei, & A. Jorres. Synthesis of C-X-C and C-C chemokines by human peritoneal fibroblasts: induction by macrophage- derived cytokines. *Am J Pathol* 158, 1441-1450 (2001)
53. Visser C.E., J. Tekstra, J.J. Brouwer-Steenbergen, C.W. Tuk, D.M. Boorsma, S.C Sampat-Sardjoepersad, S. Meijer, R.T. Krediet, & R.H. Beelen. Chemokines produced by mesothelial cells: huGRO-α, IP-10, MCP-1 and RANTES. *Clin Exp Immunol* 112, 270-275 (1998)
54. McColl SR, Clark-Lewis I. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J Immunol*.163,2829-2835 (1999)
55. Haslinger B, S. Mandl-Weber, A. Sellmayer, & T. Sitter. Hyaluronan fragments induce the synthesis of MCP-1 and IL-8 in cultured human peritoneal mesothelial cells. *Cell Tissue Res* 305, 79-76 (2001)
56. Ishihara K, Y. Yamaguchi, K. Okabe, & M. Ogawa. Neutrophil elastase enhances macrophage production of

chemokines in receptor-mediated reaction. *Res Commun Mol Pathol Pharmacol* 103,139-147 (1999)
57. Lee A, M.B.K. Whyte, & C. Haslett. In hibition of opoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leuk Biol* 54,283-288 (1993)
58. Savill J, V. Fadok, P. Hersar & C. Haslett. Phagocyte recognition of cell undergoing apoptosis. *Immunol Today* 14.131-136 (1993)
59. Savill J, I. Dransfield, N. Hogg, & C. Haslett. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* 343, 170-173 (1990)
60. Collins M.K, G.R. Perkins, G. Rodriguez-Tarduchy, M.A. Nieto, & A. Lopez-Rivas. Growth factors as survival factors: Regulation of apoptosis. *Bioassays* 16,133-138 (1994)
61. Hegyi L, S.J. Hardwick, R.C. Siow, & J.N. Skepper. Macrophage death and the role of apoptosis in human atherosclerosis. *J Hematother Stem Cell Res* 10,27-42 (2001)
62. Cockerill GW, J.R. Gamble, & M.A. Vadas. Angiogenesis: Models and modulators. *Internatl Rev Cytol* 159,113-160 (1995)
63. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280,C1358-1366 (2001)
64. Shibuya M. Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Struct Funct* 26,25-35 (2001)
65. Szekanecz Z., & A.E. Koch. Chemokines and angiogenesis. *Curr Opin Rheumatol* 13:202-208 (2001)
66. Kurz H. Physiology of angiogenesis. *J Neurooncol* 50,17-35 (2000)
67. Parks W.C. Matrix metalloproteinases in repair. *Wound Repair Regen* 7,423-432 (1999)
68. Vincenti M.P. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. *Methods Mol Biol* 151,121-148 (2001)
69. Lund L.R., J. Romer, T.H. Bugge, B.S. Nielsen, T.L. Frandsen, J.L. Degen, R.W. Stephens, & K Dano. Functional overlap between two classes of matrix-degrading proteases in wound healing. *EMBO J* 18,4645-4656 (1999)
70. Gomez D.E, A.F. Alonso, H. Yoshiji, & U.P. Torgeirsson. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 74,111-122 (1997)
71. Tonnesen M.G, X. Feng, & R.A. Clark. Angiogenesis in wound healing. *J Investig Dermatol Symp Proc* 5, 40-46 (2000)
72. Takehara K. Growth regulation of skin fibroblasts. *J Dermatol Sci* 24(Suppl): S70-77 (2000)
73. Cross M.J, & L Claesson-Welsh. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci* 22, 201-207 (2001)
74. Ornitz D.M., & N. Itoh. Fibroblast growth factors. *Genome Biol* 2, 3005 (2001)
75. Ma C., R.W. Tarnuzzer & N. Chegini. Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in mesothelial cells and their regulation by transforming growth factor-beta1. *Wound Repair Regen* 7, 477-485 (1999)
76. Chegini N. K. Kotseos, Y. Zhao, C. Ma, F. McLean, M.P. Diamond, L. Holmdahl, & J. Burns. The expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in serosal tissue of intraperitoneal organs and adhesions. *Fertil Steril* 76, 1212-1219 (2001)
77. Chegini N, K. Kotsesos, B. Bennett, F.W. McLean, M.P. Diamond, L. Holmdahl & J. Burns. Matrix Metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in peritoneal fluids and sera, and correlation with peritoneal adhesions. *Fertil Steril* 76, 1207-1211(2001)
78. Sharpe-Timms K.L., R.L. Zimmer, W.J. Jolliff, J.A. Wright, W.B. Nothnick, & T.E. Curry. Gonadotropin-releasing hormone agonist (GnRH-a) therapy alters activity of plasminogen activators, matrix metalloproteinases, and their inhibitors in rat models for adhesion formation and endometriosis: potential GnRH-a-regulated mechanisms reducing adhesion formation. *Fertil Steril* 69, 916-923 (1998)
79. Wiczyk H.P, D.R. Grow, L.A. Adams, D.L. O'Shea, & M.T. Reece. Pelvic adhesions contain sex steroid receptors and produce angiogenesis growth factors. *Fertil-Steril* 69, 511-516 (1998)
80. Rout U.K, K. Oommen & M.P. Diamond. Altered expressions of VEGF mRNA splice variants during progression of uterine-peritoneal adhesions in the rat. *Am J Reprod Immunol* 43, 299-304 (2000)
81. Howdieshell T.R, C. Riegner, V. Gupta, D. Callaway, K. Grembowicz, & P.L. Sathyanarayana-McNeil. Normoxic wound fluid contains high levels of vascular endothelial growth factor. *Ann Surg* 228, 707-715 (1998)
82. Swift M.E, H.K. Kleinman, & L.A. DiPietro. Impaired wound repair and delayed angiogenesis in aged mice. *Lab Invest* 79, 1479-1487 (1999)
83. O'Reilly M.S, L. Holmgren, Y. Shing. C. Chen, R.A. Rosenthal, M. Moses, W.S. Lane, Y Cao, E.H. Sage, & J Folkman. Angiostatin: A novel angiogenesis inhibitor that modulates the suppression of metastases by a Lewis Carcinoma. *Cell* 79, 315-328 (1994)
84. Fotsis T., M.S. Pepper, H. Aldercrutz, G. Fleischmann T. Hase , R. Montesano & L. Schweigerer. Genistein, a deitry-derived inhibitor of in vivo angiogenesis. *Proc Natl Acad Sci USA* 90, 2690-2694 (1993)
85. Fotsis T, Y. Zhang, M.S. Pepper, H. Adlercreutz, R. Montesano, P.P. Nawroth, & L. Schweigerer. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppress tumor growth. *Nature* 368, 237-239 (1994)
86. Liekens S, E. De Clercq, & J. Neyts. Angiogenesis: regulators and clinical applications. *Biochem Pharmacol* 61,253-270 (2001)
87. Chiang S.C, C.H. Cheng, K.S. Moulton, J.M. Kasznica, & S.L. Moulton. TNP-470 inhibits intraabdominal adhesion formation. *J Pediatr Surg* 35, 189-196 (2000)
88. Chegini N., M.J. Rossi, G.S. Schultz, W.A. Dunn Jr. & B. J. Masterson. Cellular distribution of EGF, TGF-α and EGF receptor in fascia and peritoneum during healing: An autoradiographic and immunocytochemical study. *Wound Repair Reg* 1, 27-40 (1993)
89. Chegini N., J. Simms, R.S. Williams, & B.J. Masterson. Identification of epidermal growth factor, transforming

growth factor-a, and epidermal growth factor receptor in surgically induced pelvic adhesions in the rat and intraperitoneal adhesions in the human. *Am J Obstet Gynecol* 171, 321-328 (1994)
90. Faull R.J, J.M. Stanley, S. Fraser, D.A. Power, & D.I. Leavesley. HB-EGF is produced in the peritoneal cavity and enhances mesothelial cell adhesion and migration. *Kidney Int.* 59, 614-624 (2001)
91. Chegini N. The effect of growth factors on peritoneal cell growth. (perssonal observations)
92. Howdieshell T.R, D. Callaway, W.L. Webb, M.D. Gaines, C.D. Procter Jr, Sathyanarayana, J.S. Pollock, T.L. Brock, & P.L. McNeil. Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation. *J Surg Res* 96, 173-182 (2001)
93. Jones J., & D. Clemmons. Insulin-like growth factors and their binding proteins, biological actions. *Endocrine Rev* 16, 3-34 (1994)
94. Chegini N., X-M. Tang, M.J. Rossi, S. Barnes, C.J.M. Kane, & B.J. Masterson. Detection of relaxin in rat and human Skin wound and its effect in skin fibroblast in vitro. *Wound Repair Reg.* Abst #33 (1993)
95. Gimbel M.L, D. Chelius, T.K. Hunt, & E.M. Spencer. A novel approach to reducing postoperative intraperitoneal adhesions through the inhibition of insulin like growth factor I activity. *Arch Surg* 136, 311-317 (2001)
96. Campbell P.G., S.K. Durham, J.D. Hayes, A. Suwanichkul, & D.R. Powell. Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. *J Biol Chem* 274, 30215-30221 (1999)
97. Gibson T.L, & P Cohen. Inflammation-related neutrophil proteases, cathepsin G and elastase, function as insulin-like growth factor binding protein proteases. *Growth Horm IGF Res* 9, 241-253 (1999)
98. Herouy Y., P. Mellios, E. Bandemir, C Stetter, S. Dichmann, M. Idzko, C. Hofmann, W. Vanscheidt, E. Schopf, & J. Norgauer. Autologous platelet-derived wound healing factor promotes angiogenesis via $\alpha v\beta 3$-integrin expression in chronic wounds. *Int J Mol Med* 6, 515-519 (2000)
99. Chegini N., K. Kotseos, C. Ma, R.S. Williams, M.P. Diamond, L. Holmdahl & K Skinner. Differential expression of integrin αv and $\beta 3$ in serosal tissue of human intraperitoneal organs and adhesion. *Fertil Steril* 75, 791-796 (2001)
100. Tietze L., J. Borntraeger, B. Klosterhalfen, B. Amo-Takyi, S. Handt, K. Gunther, & S Merkelbach-Bruse. Expression and function of $\beta 1$ and $\beta 3$ integrins of human mesothelial cells in vitro. *Exp Mol Pathol* 66, 131-139 (1999)
101. Witz C.A, I.A. Montoya-Rodriguez,, D.M. Miller, B.G. Schneider, & R.S. Schenken. Mesothelium expression of integrins in vivo and in vitro. *J Soc Gynecol Investig* 5, 87-93 (1998)
102. Arroyo A.G., D. Taverna, C.A. Whittaker, U.G. Strauch, B.L. Bader, H. Rayburn, D. Crowley, C.M. Parker, & R.O Hynes. In vivo roles of integrins during leukocyte development and traffic: insights from the analysis of mice chimeric for $\alpha 5$, αv, and $\alpha 4$ integrins. *J Immunol* 165, 4667-4675 (2000)
103. Sheppard D. Integrin-mediated activation of transforming growth factor-beta(1) in pulmonary fibrosis. *Chest* 120(Suppl): 49S-53S (2001)
104. Sime P.J, & K.M. O'Reilly. Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. *Clin Immunol* 99, 308-319 (2001)
105. Murphy-Ullrich J.E, & M. Poczatek. Activation of latent TGF-β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* 11,59-69 (2000)
106. Koli K, J. Saharinen, M. Hyytiainen, C. Penttinen, & J. Keski-Oja. Latency, activation, and binding proteins of TGF-β. *Microsc Res Tech* 52,354-362 (2001)
107. Dou, Q., R.S. Williams & N. Chegini. The expression of integrin messenger ribonucleic acid in human endometrium; a quantitative reverse transcription polymerase chain reaction study. *Fertil. Streil* 71, 347-353 (1999)
108. Singer A.J & R.A. Clark. Cutaneous wound healing. N *Engl J Med* 341,738-746 (1999)
109. Saed G.M, W. Zhang, N. Chegini, L. Holmdahl, & M.P. Diamond. Alteration of type I and III collagen expression in human peritoneal mesothelial cells in response to hypoxia and transforming growth factor-beta1. *Wound Repair Regen* 7, 504-510 (1999)
110. Branton M.H., & J.B. Kopp. TGF-β and fibrosis. *Microbes Infect* 1, 1349-1365 (1999)
111. Koch R.M., N.S. Roche, W.T. Parks, G.S. Ashcroft J.J. Letterio, & A.B. Roberts. Incisional wound healing in transforming growth factor-$\beta 1$ null mice. *Wound Repair Regen* 8, 179-191 (2000)
112. Crowe M.J., T Doetschman, & D.G. Greenhalgh. Delayed wound healing in immunodeficient TGF-$\beta 1$ knockout mice. *J Invest Dermatol* 115, 3-11 (2000)
113. Tyrone J.W, J.R. Marcus, S.R. Bonomo, J.E. Mogford, Y. Xia, & T.A. Mustoe. Transforming growth factor $\beta 3$ promotes fascial wound healing in a new animal model. *Arch Surg* 135,1154-1159 (2000)
114. Chegini N, K. Kotseos, Y. Zhao, B. Bennett, F.W. McLean, M.P. Diamond, L. Holmdahl, & J. Burns. Differential expression of TGF-$\beta 1$ and TGF-$\beta 3$ in serosal tissues of human intraperitoneal organs and peritoneal adhesions. *Hum Reprod* 6, 1291-300 (2001)
115. Chegini N, H. Rong, B. Bennett, & I.K. Stone. Peritoneal fluid cytokine and eicosanoid levels and their relation to the incidence of peritoneal adhesion. *J Soc Gynecol Investig* 6,153-157 (1999)
116. Holmdahl L, K. Kotseos, M. Bergstrom, P. Falk, M.L. Ivarsson, & N Chegini. Overproduction of transforming growth factor-beta1 (TGF-$\beta 1$) is associated with adhesion formation and peritoneal fibrinolytic impairment. *Surgery* 129,26-32 (2001)
117. Chegini N., L.I. Gold, R.S. Williams, & B.J. Masterson. Localization of transforming growth factor beta isoforms TGF-$\beta 1$, TGF-$\beta 2$, and TGF-$\beta 3$ in surgically induced pelvic adhesions in the rat. *Obstet Gynecol* 83, 449-454 (1994)
118. Chegini N. The role of growth factors in peritoneal healing: transforming growth factor beta (TGF-β) Eur J Surg (Suppl) 577,17-22 (1997)
119. Ghellai A.M., A.F. Stucchi, N. Chegini, C. Ma, C.D. Andry, J.M. Kaseta, J.W. Burns, K.C. Skinner, & J.M. Becker. Role of transforming growth factor beta-1 in peritonitis-induced adhesions. *J Gastrointest Surg* 4,316-23 (2000)
120. Rong H, X-M.Tang, Y. Zhao, S.C. Juneja, M.F. Fay, R.S. Williams & N. Chegini. Peritoneal exposure to surgical glove powders differentially modulate the inflammatory and immune related cytokines production during surgically-induced adhesion formation. *Wound Repair Reg* 5, 89-96 (1997)

121. Lucas P.A., D.J. Warejcka, H.E. Young, & B.Y. Lee. Formation of abdominal adhesions is inhibited by antibodies to transforming growth factor-beta1. *J Surg Res* 65,135-138 (1996)
122. Williams R.S, A.M. Rossi, N. Chegini, & G. Schultz. Effect of transforming growth factor beta on postoperative adhesion formation and intact peritoneum. *J Surg Res* 52, 65-70 (1992)
123. Krause T.J, D. Katz, C.J. Wheeler, S. Ebner, & R.D. McKinnon. Increased levels of surgical adhesions in TGFb1 heterozygous mice. *J Invest Surg* 12,31-38 (1999)
124. Lanfrancone L., D. Boraschi, P. Ghiara, B. Falini, F. Grignani, G. Peri, A. Mantovani, & P.G. Pelicci. Human peritoneal mesothelial cells produce many cytokines (granulocyte colony-stimulating factor (CSF), granulocyte-monocyte-CSF, macrophage-CSF, interleukin-1 (IL-1), and IL-6) and are activated and stimulated to grow by IL-1. *Blood* 80, 2835-2842 (1992)
125. D.W. H. Riches. Macrophages involvement in wound repair, remodeling and fibrosis. In: The molecular and cellular biology of wound repair. Ed. R.A.F. Clark 2nd ed New York: Plenum Press, 95-141 (1996)
126. Niessen F.B, M.P. Andriessen, J. Schalkwijk, L. Visser, & W Timens. Keratinocyte-derived growth factors play a role in the formation of hypertrophic scars. *J Pathol* 194, 207-216 (2001)
127. Ma C, Q. Duo & N Chegini. Modulation of interleukin (IL), IL-2, IL-5, IL-6, IL-12, IL-13 and IL-16 expression in human mesothelial cells, macrophage and lymphocyte exposed to glove powders, latex proteins and LPS. *Fertil Steril.* 74, Abst. # P892 (1998)
128. Shadrin O.V, O.Y. Kaufman, O.M. Dronova, & S.N. Bykovskaya. Recombinant interleukin-2 significantly increases the survival of mice with peritonitis, but not acute Staphylococcus aureus peritoneal infection. *Mol Biother* 4, 151-154 (1992)
129. Cohen M.C, & S. Cohen. Cytokine Function. A study in biologic diversity. *Am J Clin Pathol* 105, 589-598 (1996)
130. Miossec P. Interleukin 4. A potential anti-inflammatory agent. *Rev Rheum* 60, 119-124 (1993)
131. Beckmann M.P, D. Cosman, W. Fanslow, C.R. Maliszewski, & S.D. Lyman. The interleukin-4 receptor: structure, function, and signal transduction. *Chem Immunol* 51, 107-134 (1992)
132. Salmon-Ehr V., L. Ramont, G. Godeau, P. Birembaut, M. Guenounou, P. Bernard, & F.X Maquart. Implication of interleukin-4 in wound healing. *Lab Invest* 80, 1337-1343 (2000)
133. Hirano T. The biology of interleukin-6. Chem *Immunol* 51, 153-180 (1992)
134. Donnelly RP, L.J. Crofford, S.L. Freeman, J. Buras, E. Remmers, R.L. Wilder, & M.J. Fenton.Tissue-specific regulation of IL-6 production by IL-4. Differential effects of IL-4 on nuclear factor-kappa B activity in monocytes and fibroblasts. *J Immunol* 151, 5603-5612 (1993)
135. Saba A.A., A.A.Kaidi, V. Godziachvili, G.W. Dombi, E.J. Dawe, J.H. Libcke, & Y.J. Silva. Effects of interleukin-6 and its neutralizing antibodies on peritoneal adhesion formation and wound healing. *Am Surg* 62,569-572 (1996)
136. Gallucci R.M, P.P. Simeonova, J.M. Matheson, C. Kommineni, J.L. Guriel, T. Sugawara, & M.I. Luster. Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. *FASEB J* 14,2525-2531 (2000)
137. Arici A., S.I. Tazuke, E. Attar, H.J. Kliman, & D.L. Olive. Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. *Mol Hum Reprod* 2, 40-45 (1996)
138. Iocono J.A, K.R. Colleran, D.G. Remick, B.W. Gillespie, H.P. Ehrlich, & W.L. Garner. Interleukin-8 levels and activity in delayed-healing human thermal wounds. *Wound Repair Regen* 8, 216-225 (2000)
139. Rennekampff H.O., J.F. Hansbrough, V. Kiessig, C. Dore, M. Sticherling, & J.M. Schroder. Bioactive interleukin-8 is expressed in wounds and enhances wound healing. *J Surg Res* 93,41-54 (2000)
140. Holzheimer R.G, & W. Steinmetz. Local and systemic concentrations of pro- and anti-inflammatory cytokines in human wounds. *Eur J Med Res* 5,347-355 (2000)
141. Cavaillon J.M. Pro- versus anti-inflammatory cytokines: myth or reality. *Cell Mol Biol* 47, 695-702 (2001)
142. Sato Y, T. Ohshima, & T. Kondo. Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing. *Biochem Biophys Res Commun* 265,194-199 (1999)
143. Holschneider C.H., P.M. Cristoforoni, K. Ghosh, M. Punyasavatsut, E. Abed, & F.J. Montz. Endogenous versus exogenous IL-10 in postoperative intraperitoneal adhesion formation in a murine model. *J Surg Res* 70, 138-143 (1997)
144. Sewnath M.E, D.P. Olszyna, R. Birjmohun, F.J. ten Kate, D.J. Gouma, & T. vanDer Poll. IL-10-deficient mice demonstrate multiple organ failure and increased mortality during Escherichia coli peritonitis despite an accelerated bacterial clearance. *J Immunol* 166, 6323-6331 (2001)
145. Zingarelli B., Z. Yang, P.W. Hake, A. Denenberg, & H.R. Wong. Absence of endogenous interleukin 10 enhances early stress response during post-ischaemic injury in mice intestine. *Gut* 48: 610-622 (2001)
146. Opal S.M, & V.A. DePalo. Anti-inflammatory cytokines. *Chest* 117, 1162-1172 (2000)
147. Scott P. IL-12: initiation cytokine for cell-mediated immunity. *Science* 260, 496-507 (1993)
148. Steinhauser ML, C.M. Hogaboam, N.W. Lukacs, R.M. Strieter, & S.L. Kunkel. Multiple roles for IL-12 in a model of acute septic peritonitis. *J Immunol* 162, 5437-5443 (1999)
149. Brombacher F. The role of interleukin-13 in infectious diseases and allergy. *Bioessays* 22,646-56 (2000)
150. Chomarat, P. & J. Banncherеau. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int Rev Immunol* 17, 1-52 (1998)
151. Waldmann T.A, & Y. Tagaya. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens1. *Annu Rev Immunol* 17, 19-49 (1999)
152. Fehniger T.A, & M.A. Caligiuri. Interleukin 15: biology and relevance to human disease. *Blood* 97,14-32 (2001)
153. Marks-Konczalik J., S. Dubois, J.M. Losi, H. Sabzevari, N. Yamada, L. Feigenbaum, T.A. Waldmann, &

Y Tagaya. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc. Natl. Acad. Sci. USA* 97, 11445-11450 (2000)

154. Chegini N, M. Roberts, B.A. Ripps. Differential expression of interlukines (IL) IL-13 and IL-15 in endometrial implants and peritoneal fluids of women with endometriosis. *J Reprod Immunol* 2002.

155. Swain S.L. Interleukin 18: tipping the balance towards a T helper cell 1 response. *J Exp Med* 194, F11-4 (2001)

156. Kampfer H., H. Muhl, M. Manderscheid, U. Kalina, D. Kauschat, J. Pfeilschifter, & S Frank. Regulation of interleukin-18 (IL-18) expression in keratinocytes (HaCaT): implications for early wound healing. *Eur Cytokine Netw* 11, 626-633 (2000)

157. Kampfer H., J. Paulukat, H. Muhl, C Wetzler, J. Pfeilschifter, & S Frank. Lack of interferon-gamma production despite the presence of interleukin-18 during cutaneous wound healing. *Mol Med* 6,1016-1027 (2000)

158. Ma X. TNF-α and IL-12: a balancing act in macrophage functioning. *Microbes Infect* 3, 121-129 (2001)

159. Gillitzer R, & M. Goebeler. Chemokines in cutaneous wound healing. *J Leukoc Biol* 69, 513-521 (2001)

160. Engelhardt E, A. Toksoy, M. Goebeler, S. Debus, E.B. Brocker, & R Gillitzer. Chemokines IL-8, GROα, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol* 153, 1849-1860 (1998)

161. Jackman S.H., M.B. Yoak, S. Keerthy, & B.L. Beaver. Differential expression of chemokines in a mouse model of wound healing. *Ann Clin Lab Sci.* 30, 201-207 (2000)

162. Wetzler C, H. Kampfer, J. Pfeilschifter, & S. Frank. Keratinocyte-derived chemotactic cytokines: expressional modulation by nitric oxide in vitro and during cutaneous wound repair in vivo. *Biochem Biophys Res Commun* 274: 689-696 (2000)

163. Low Q.E., I.A. Drugea, L.A. Duffner, D.G. Quinn, D.N. Cook, B.J. Rollins, E.J. Kovacs, & L.A. DiPietro. Wound healing in MIP-1a (-/-) and MCP-1(-/-) mice. *Am J Pathol* 159, 457-463 (2001)

164. Luster A.D., R.D. Cardiff, J.A. MacLean, K. Crowe, & R.D. Granstein. Delayed wound healing and disorganized neovascularization in transgenic mice expressing the IP-10 chemokine. *Proc Assoc Am Physicians.* 110: 183-196 (19998)

165. Shiraha H., A. Glading, K. Gupta, & A. Wells. IP-10 inhibits epidermal growth factor-induced motility by decreasing epidermal growth factor receptor-mediated calpain activity. *J Cell Biol* 146, 243-254 (1999)

166. Nanney L.B, S.G. Mueller, R. Bueno, S.C. Peiper, & A. Richmond. Distributions of melanoma growth stimulatory activity of growth-regulated gene and the interleukin-8 receptor B in human wound repair. *Am J Pathol* 147, 1248-1260 (1995)

167. Nirodi C.S, R. Devalaraja, L.B. Nanney, S Arrindell, S. Russell, J. Trupin, & A. Richmond. Chemokine and chemokine receptor expression in keloid and normal fibroblasts. *Wound Repair Regen* 8,371-382 (2000)

168. Zeyneloglu H.B., E Seli, L.M. Senturk, L.S. Gutierrez, D.L. Olive, A. Arici. The effect of monocyte chemotactic protein 1 in intraperitoneal adhesion formation in a mouse model. *Am J Obstet Gynecol* 179, 438-443 (1998)

169. Mrstik M, K. Kotseos C. Ma & N. Chegini. Increased expression of interferon-gamma inducible protein-10 (IP-10) mRNA during surgically-induced peritoneal injury. *J Soc Gynecol Invest* ,7;Abst # 828 (2000)

170. Zeyneloglu H.B, L.M Senturk, E. Seli, E. Oral, D.L. Olive & A. Arici. The role of monocyte chemotactic protein-1 in intraperitoneal adhesion formation. *Hum Reprod* 13, 1194-1199 (1998)

171. Gallo R.L. Proteoglycans and cutaneous vascular defense and repair. *J Investig Dermatol Symp Proc* 5,55-60 (2000)

172. Yamada K.M, J. Gailit, & R.A. F. Clark. Integrin in wound repair. In: The molecular biology of wound repair. Ed. R.A.F. Clark. 2nd ed. New York: Plenum Press, 311-330 (1996)

173. Idell S., C. Zwieb, J. Boggaram, D. Holiday, A.R. Johnson, & G. Raghu. Mechanisms of fibrin formation and lysis by human lung fibroblasts: influence of TGF-b and TNF-*a. Am J Physiol.* 263: L487-494 (1992)

174. Keene D.R., M.P. Marinkovich, & L.Y. Sakai. Immunodissection of the connective tissue matrix in human skin. *Microsc Res Tech* 38, 394-406 (1997)

175. Iozzo R.V., & A.D. Murdoch. Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. *FASEB* 10,598-614 (1996)

176. Raghow R. The role of extracellular matrix in post-inflammatory wound healing and fibrosis. *FASEB* 8,823-831 (1994)

177. Hu B., Y.L. Kapila, M. Buddhikot, M. Shiga, & S. Kapila. Coordinate induction of collagenase-1, stromelysin-1 and urokinase plasminogen activator (uPA) by the 120-kDa cell-binding fibronectin fragment in fibrocartilaginous cells: uPA contributes to activation of procollagenase-1. *Matrix Biol.*19,657-669 (2000)

178. Olman M.A, J.S. Hagood, W.L. Simmons, G.M. Fuller, C. Vinson, & K.E. White. Fibrin fragment induction of plasminogen activator inhibitor transcription is mediated by activator protein-1 through a highly conserved element. *Blood* 94, 2029-2038 (1999)

179. Saed G., W. Zhang, K.A. Wie, F.D. Yelian, & M.P. Diamond. The effect of TGF-β and hypoxia on fibronectin expression. *J Soc Gynecol Invest* 7, 348-354 (2000)

180. Jang Y.C., R. Tsou, N.S. Gibran, & F.F. Isik. Vitronectin deficiency is associated with increased wound fibrinolysis and decreased microvascular angiogenesis in mice. *Surgery* 127, 696-704 (2000)

181. Shaw R.J, D.E. Doherty, A.G. Ritterl, S.H. Benedict & R.A. Clark. Adherance-dependent increase in human monocyte PDGF(B) mRNA is associated with increases in c-fos, c-jun and EGR-2 mRNA. *J Cell Biol* 111, 2139-2148 (1990)

182. Gharaee-Kermani M, & S.H. Phan. Role of cytokines and cytokine therapy in wound healing and fibrotic diseases. *Curr Pharm Des.*7, 1083-1103 (2001)

183. Ramanathan M. A pharmacokinetic approach for evaluating cytokine binding macromolecules as antagonists. *Pharm Res* 13, 84-90 (1996)

184. Arend W.P. Inhibiting the effects of cytokines in human diseases. Adv Intern Med 40, 365-394 (1995)

185. Gehr G, T. Braun, & W. Lesslauer. Cytokines, receptors, and inhibitors. *Clin Invest* 70, 64-69 (1992)

186. Holmdahl L. The role of fibrinolysis in the formation of adhesions. *Eur J Surg* (Suppl) 577, 24-31 (1997)
187. Falk P., C. Ma, N. Chegini, & L. Holmdahl. Differential regulation of mesothelial cell fibrinolysis by transforming growth factor beta 1. *Scand J Clin Lab Invest* 60, 439-47 (2000)
188. Bugge T.H., M.J. Flick, M.J. Danton, C.C. Daugherty, J. Romer, K. Dano, P. Carmeliet, D. Collen, & J.L. Degen. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. *Proc Natl Acad Sci USA*. 93,5899-904 (1996)
189. Bugge T.H, K.W. Kombrinck, M.J. Flick, C.C. Daugherty, M.J. Danton, & J.L. Degen. Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell* 87, 709-719 (1996)
190. Carmeliet P, & D. Collen. Molecular genetics of the fibrinolytic and coagulation systems in haemostasis, thrombogenesis, restenosis and atherosclerosis. *Curr Opin Lipidol* 8,118-125 (1997)
191. Horton M.R, M.A. Olman, & P.W Noble. Hyaluronan fragments induce plasminogen activator inhibitor-1 and inhibit urokinase activity in mouse alveolar macrophages: a potential mechanism for impaired fibrinolytic activity in acute lung injury. *Chest* 116 (Suppl),17S (1999)
192. Fitzpatrick S.L, G. Kassam, K.S. Choi, H.M. Kang, D.K. Fogg, & D.M. Waisman. Regulation of plasmin activity by annexin II tetramer. *Biochemistry* 39,1021-1028 (2000)
193. Holmdahl L, E. Eriksson, B.I. Eriksson, & B. Risberg. Depression of peritoneal fibrinolysis during surgery is a local response to trauma. *Surgery* 123,539-544 (1998)
194. Tsuji K, Y. Eguchi, & M. Kodama. Postoperative hypercoagulable state followed by hyperfibrinolysis related to wound healing after hepatic resection. *J Am Coll Surg* 183,230-238 (1996)
195. Lai H.S, Y. Chen, K.J. Chang, & W.J. Chen. Tissue plasminogen activator reduces intraperitoneal adhesion after intestinal resection in rats. *J Formos Med Assoc* 97, 323-327 (1998)
196. Montz F.J, J.M. Fowler, A.J. Wolff, S.M. Lacey, & M. Mohler. The ability of recombinant tissue plasminogen activator to inhibit post-radical pelvic surgery adhesions in the dog model. *Am J Obstet Gynecol* 165, 1539-1542 (1991)
197. Dunn R.C, & M. Mohler. Effect of varying days of plasminogen activator therapy on the prevention of postsurgical adhesions in a rabbit model. *J Surg Res* 54, 242-245 (1993)
198. Evans D.M, K. McAree, D.P. Guyton, N. Hawkins, & K. Stakleff. Dose dependency and wound healing aspects of the use of tissue plasminogen activator in the prevention of intra-abdominal adhesions. *Am J Surg* 165, 229-232 (1993)
199. Arumugam S., Y.C. Jang, C. Chen-Jensen, N.S. Gibran, & F.F. Isik. Temporal activity of plasminogen activators and matrix metalloproteinases during cutaneous wound repair. *Surgery* 125,587-593 (1999)
200. Falk K., P. Bjorquist, M. Stromqvist, & L. Holmdahl. Reduction of experimental adhesion formation by inhibition of plasminogen activator inhibitor type 1. *Br J Surg* 88, 286-289 (2001)
201. Ravanti L., & V.M. Kahari. Matrix metalloproteinases in wound repair. *Int J Mol Med*. 6,391-407 (2000)
202. Raza S.L, & L.A. Cornelius. Matrix metalloproteinases and their native or pharmacologic inhibitors. *Adv Dermatol* 16, 185-208 (2000)
203. Soo C., W.W. Shaw, X. Zhang, M.T. Longaker, E.W. Howard, & K. Ting. Differential expression of matrix metalloproteinases and their tissue-derived inhibitors in cutaneous wound repair. *Plast Reconstr Surg* 105, 638-647 (2000)
204. von Lampe B., B. Barthel, S.E. Coupland, E.O. Riecken, S. Rosewicz. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 47, 63-73 (2000)
205. Makela M, H. Larjava, E. Pirila, P. Maisi, T. Salo, T. Sorsa, & V.J. Uitto. Matrix metalloproteinase 2 (gelatinase A) is related to migration of keratinocytes. *Exp Cell Res* 251, 67-78 (1999)
206. Lempinen M., K. Inkinen, H. Wolff, & J. Ahonen. Matrix metalloproteinases 2 and 9 in indomethacin-induced rat gastric ulcer. *Eur Surg Res* 32, 169-176 (2000)
207. Savage F.J, D.L. Lacombe, R.M. Hembry, & P.B. Boulos. Effect of colonic obstruction on the distribution of matrix metalloproteinases during anastomotic healing. *Br J Surg* 85,72-75 (1998)
208. Tarlton J.F, C.J. Vickery, D.J. Leaper, & A.J. Bailey. Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. *Br J Dermatol* 137, 506-516 (1997)
209. Agren M.S. Matrix metalloproteinases (MMPs) are required for re-epithelialization of cutaneous wounds. *Arch Dermatol Res* 291: 583-90 (1999)
210. Ashcroft G.S, M.A. Horan, S.E. Herrick, R.W. Tarnuzzer, G.S. Schultz, & M.W. Ferguson. Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. *Cell Tissue Res* 290, 581-591 (1997)
211. Pirila E., N. Ramamurthy, P. Maisi, S. McClain, A. Kucine, J. Wahlgren, L. Golub, T. Salo, & T Sorsa. Wound healing in ovariectomized rats: effects of chemically modified tetracycline (CMT-8) and estrogen on matrix metalloproteinases -8, -13 and type I collagen expression. *Curr Med Chem* 8, 281-294 (2001)
212. Agren M.S, L.N. Jorgensen, M. Andersen, J. Viljanto, & F. Gottrup. Matrix metalloproteinase 9 level predicts optimal collagen deposition during early wound repair in humans. *Br J Surg* 85, 68-71 (1998)
213. Mirastschijski U, M.A. Karsdal, B. Jeppsson, & M.S. Agren. Matrix metalloproteinses (MMPs) in epidermal and peritoneal wound healing. *Wound Repair Reg*, 6 Abst #A321 (1998)
214. Syk I., M.S. Agren, D. Adawi, & B. Jeppsson. Inhibition of matrix metalloproteinases enhances breaking strength of colonic anastomoses in an experimental model. *Br J Surg* 88,228-234 (2001)
215. Liekens S., E. De Clercq, & J. Neyts. Angiogenesis: regulators and clinical applications. *Biochem Pharmacol* 61,253-270 (2001)
216. Klimp AH, E. van der Vaart, P.O. Lansink, S. Withoff, E.G. de Vries, G.L. Scherphof, J. Wilschut, & T.

Daemen. Activation of peritoneal cells upon in vivo transfection with a recombinant alphavirus expressing GM-CSF. *Gene Ther* 8, 300-307 (2001)
217. Hoff C.M. Ex vivo and in vivo gene transfer to the peritoneal membrane in a rat model. *Nephrol Dial Transplant* 16,666-668 (2001)
218. Jackman R.W., T.D. Stapleton, E.M. Masse, V.S. Harvey, M.S. Meyers, T.R. Shockley, & J.A. Nagy. Enhancement of the functional repertoire of the rat parietal peritoneal mesothelium in vivo: directed expression of the anticoagulant and anti-inflammatory molecule thrombomodulin. *Hum Gene Ther* 9,1069-1081 (1998)
219. Jiang A, & R. Dornburg. In vivo cell type-specific gene delivery with retroviral vectors that display single chain antibodies. *Gene Ther* 6,1982-1987 (1999)
220. Murphy J.E., & J.G. Rheinwald. Intraperitoneal injection of genetically modified human mesothelial cells for systemic gene therapy. *Hum Gene Ther* 8,1867-1879 (1997)

Key Words: Peritoneum, Wound Repair, Adhesions, Growth Factors, Cytokines, Chemokines, Proteases, Clinical, Molecular, Review

Send correspondence to: Dr. Nasser Chegini, Department of Obstetrics and Gynecology, University of Florida, Gainesville, FL, 32610, Tel: 352-392-3929, Fax: 352-392-6994, E-mail: cheginin@obgyn.ufl.edu

Exhibit C

Original article

Reduction of experimental adhesion formation by inhibition of plasminogen activator inhibitor type 1

K. Falk, P. Björquist*, M. Strömqvist† and L. Holmdahl

Department of Surgery, Sahlgrenska University Hospital, Göteborg University, Göteborg, and Departments of *Cell Biology and Biochemistry and †Molecular Biology, AstraZeneca Research and Development, Mölndal, Sweden

Correspondence to: Dr L. Holmdahl, Department of Surgery, Sahlgrenska University Hospital/Östra, Göteborg University, S-41685 Göteborg, Sweden (e-mail: lena.holmdahl@surgery.gu.se)

Background: Adhesion formation is a common cause of complications following surgery. A reduction in peritoneal fibrinolytic capacity during operation is a key mechanism in the early formation of adhesions. An increase in the main inhibitor of fibrinolysis, plasminogen activator inhibitor type 1 (PAI-1), is a major factor in the loss of fibrinolytic activity. The aim of this study was to investigate if inhibition of PAI-1 could reduce the formation of adhesions after surgery.

Methods: Mice ($n = 53$) were subjected to a standard surgical procedure in order to induce adhesion formation to the abdominal side wall. At the conclusion of the operation, fragments for antigen binding of polyclonal rabbit antibody against PAI-1 (PRAP-1) were injected intraperitoneally, at two different concentrations. Control animals received an equal volume of the vehicle (saline). One week after operation adhesion formation was quantified.

Results: Both doses of PRAP-1 significantly reduced adhesion formation compared with the saline control ($P = 0{\cdot}003$ and $P = 0{\cdot}002$). There were no signs of bleeding in the postoperative period or at reoperation.

Conclusion: The present observations lend further support to the hypothesis of a pivotal role of fibrinolysis in the early formation of adhesions, and open up new possibilities for adhesion reduction by inhibiting PAI-1.

Paper accepted 6 August 2000

British Journal of Surgery 2001, **88**, 286–289

Introduction

Adhesion formation is an almost inevitable result of abdominal surgery, found in more than 90 per cent of patients who have undergone one or more abdominal operations[1]. Adhesions are the commonest cause of small bowel obstruction[2] and can lead to infertility in women[3]. The risk of inadvertent enterotomy is as high as 19 per cent in operations on patients who have previously had surgery[4]. This translates clinically into adhesion-related readmissions in a substantial proportion of patients[5].

The formation of adhesions after surgery seems to be determined by the balance of fibrin deposition and degradation in an early phase of peritoneal tissue repair. When two damaged peritoneal surfaces come into apposition, fibrinous adhesions form between the surfaces. In the presence of sufficient fibrinolytic activity there is a resolution of the fibrin strands. However, if there is insufficient fibrinolytic activity, organization of the fibrin matrix and cellular and vascular ingrowth ensues, leading to fibrous bands between the structures[6].

Support for the role of local fibrinolytic capacity derives from animal experiments and observations in humans. Experimentally, decreased fibrinolytic activity correlated with adhesion formation in animals[7]. In humans, observations during operation revealed that surgery elicited a rapid decline in peritoneal fibrin degradation capacity. This decline was caused partly by a reduced expression of the fibrinolytic stimulator, tissue-type plasminogen activator (tPA), and was partly due to an increase in the main inhibitor, plasminogen activator inhibitor type 1 (PAI-1)[8,9]. Moreover, patients who developed severe adhesions after surgery had significantly raised levels of peritoneal PAI-1 compared with normal subjects[10].

Thus, the production and release of plasminogen activator inhibitors is believed to be a major factor in the loss of peritoneal fibrinolytic activity and inhibition of PAI-1 might therefore be beneficial. Until now, PAI-1 inhibition

has not been investigated for its potential to reduce adhesion formation.

PRAP-1 is a fragment for antigen binding (Fab) of a PAI-1-inhibiting polyclonal antibody. Inhibition of PAI-1 by PRAP-1 has been shown to significantly enhance fibrinolysis[11]. The aim of this study was to test the hypothesis that inhibition of PAI-1 activity could reduce adhesion formation after surgery.

Materials and methods

Animals

Fifty-three female NMRI mice weighing 25–30 g were used. The experiments were approved by the local ethics committee and animals were kept according to standard guidelines. The animals were given a standard pellet diet and water *ad libitum*, before and after surgery.

PRAP-1

PRAP-1 (Fab fragments of polyclonal rabbit PAI-1 antibody) was kindly provided by AstraZeneca Research and Development (Umeå, Sweden). PRAP-1 had been prepared from rabbits immunized with recombinant human PAI-1 produced in mammalian cells. The immunoglobulin (Ig) G fraction obtained was cleaved with papain. Fc fragments (the effector region) and uncleaved antibodies were removed by affinity chromatography on Protein A–Sepharose (Pharmacia Biotech, Uppsala, Sweden), and Fab fragments were concentrated by ultrafiltration. A Fab fragment of a control antibody IgG has been shown previously not to affect PAI-1 activity[12].

Surgical technique

Mice were anaesthetized by inhalation of methoxyflurane (Metofane; Pitman Moore, Mundelein, Illinois, USA). The peritoneal cavity was accessed via a midline incision and a 2×15 mm area of the parietal peritoneum on each lateral abdominal wall was excised. The peritoneal lesions were closed with four 4/0 sutures and the midline incision in one layer with a running 3/0 Dexon suture (Davies and Geck, St Louis, Missouri, USA). The procedure has been described further elsewhere[13].

Experimental design

At the end of the operation, 0·75 ml of a saline solution containing PRAP-1 15 mg/kg ($n = 18$) or 30 mg/kg ($n = 17$) or, as a control, saline solution only ($n = 18$) was administered intraperitoneally just before closure of the midline incision.

Evaluation

The presence of adhesions was assessed 7 days after operation. This has been demonstrated previously to be a suitable time point for this model[13–15]. The animals were given an overdose of isoflurane (Forene; Abbott Laboratories, Queensborough, England), and the abdomen was opened widely so that the peritoneal lesions created previously, and adherent structures, were exposed. The adhesion attachments were measured by an observer blinded to the previous treatment. The proportion of the peritoneal lesion they covered was calculated and expressed as a percentage of the original lesion. In addition, the number of attachments to different structures was noted. The method of measurement has been described in detail elsewhere[13].

Statistical analysis

After determining an overall difference in adhesion formation between groups with the Kruskal–Wallis test, the non-parametric Mann–Whitney U test was used to compare the groups. $P < 0{\cdot}05$ was considered statistically significant.

Results

All animals survived and all developed adhesions. There were no signs of bleeding in the postoperative period or at reoperation. There was a significant overall difference in adhesion formation between the groups ($P = 0{\cdot}002$). The area of induced peritoneal injury was covered by adhesions to a median level of 64 (95 per cent confidence interval (c.i.) 36–94, i.q.r. 23·3) per cent in control animals (*Fig. 1*). The group that received low-dose PRAP-1 had a significantly smaller area of adhesions (50 (95 per cent c.i. 18–76, i.q.r. 20) per cent; $P = 0{\cdot}003$). Similarly, the group that had high-dose PRAP-1 also had a reduction in adhesions (50 (95 per cent c.i. 12–76, i.q.r. 28·1) per cent; $P = 0{\cdot}002$).

Because adhesions affecting certain organs (e.g. small bowel, female genital tract) might be more important clinically than others, the distribution of adhesions in the peritoneal cavity was studied. There was no statistically significant difference in the rate of adhesion formation ($P = 0{\cdot}84$ to omentum, $P = 0{\cdot}094$ to bowel, $P = 0{\cdot}97$ to other sites), even though the PRAP-1-treated groups tended to have fewer attachments to bowel (*Fig. 2*).

Discussion

These results show that topical inhibition of PAI-1 can reduce postoperative adhesion formation. PRAP-1 has been shown to enhance fibrinolysis significantly both *in vitro* and

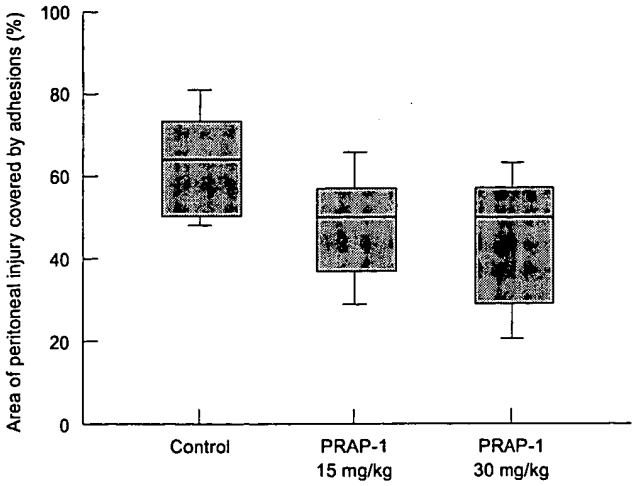


Fig. 1 Adhesion formation after surgery in mice treated with two different doses of PRAP-1 or saline control. Values are median (interquartile range) area of peritoneal injury covered by adhesions

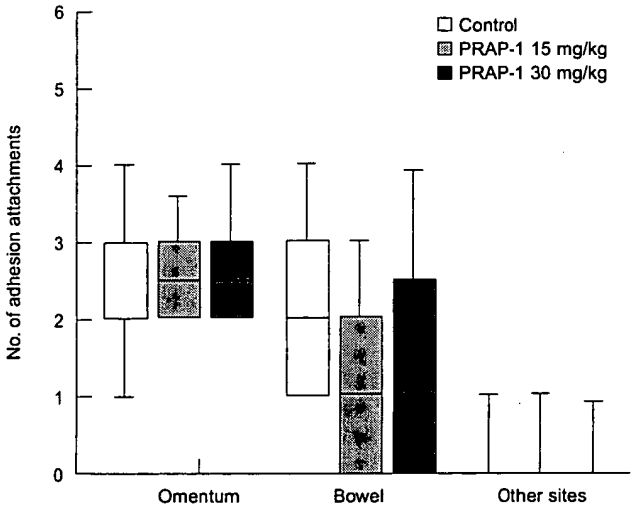


Fig. 2 Number of adhesion attachments between the peritoneal lesion and other structures. Values are median (interquartile range). PRAP-1 tended to reduce the number of attachments to bowel, but the results did not reach statistical significance ($P = 0.84$ for omentum, $P = 0.094$ for bowel, $P = 0.97$ for other sites). Kruskal–Wallis test

in vivo[11]. In rats, PRAP-1 decreased the occurrence of fibrin clots in the lungs, and reduced the thrombus size and restored blood flow in a model of arterial thrombosis[12,16]. It therefore seems reasonable to assume that the action of PRAP-1 in the peritoneal cavity was to enhance fibrinolysis.

In the present study, the low dose of PRAP-1 given intraperitoneally at the conclusion of the operation reduced the formation of adhesions, but there was no significant increase in effect when the dose was doubled. A possible explanation for this is a rapid clearance from the abdominal cavity; PRAP-1 has been demonstrated to stimulate fibrin degradation in a dose-dependent manner[12]. Compounds instilled in the abdominal cavity are known to have a short duration of action if not administered in a slow-release form[17]. The concentrations used could have saturated the abdominal cavity, so that excess PRAP-1 was absorbed by the surrounding tissues. Delivering the antibody in a slow-release form could therefore be a way of increasing its efficacy.

There are significant interspecies differences in the molecular sequence of PAI-1, which might also have affected the results. In this case a rabbit antibody against human PAI-1 was used in a mouse model. In previous studies PRAP-1 was approximately 25 times more potent in inhibiting human PAI-1 than rodent PAI-1[12]. Interspecies differences might explain the limited effect of PAI-1 in the mouse model. Although PRAP-1 significantly reduced adhesion formation, it did not alter the distribution of adhesions.

A substantial part of the fibrinolytic reduction in conditions associated with adhesion formation (e.g. surgery, inflammation, severe adhesion formation) has been observed to be caused by peritoneal overexpression of PAI-1[9,10]. Theoretically, inhibition of PAI-1 is a more controllable and biological approach to increasing local fibrinolytic capacity. Because availability of tPA would then be a rate-limiting factor, local inhibition of PAI-1 might lessen the risk of bleeding complications. However, it cannot be ruled out that systemic absorption of PRAP-1 could lead to bleeding.

The present observations lend further support to the hypothesis of a pivotal role of fibrinolysis in the early formation of adhesions, and open up new possibilities for reducing adhesions by inhibiting PAI-1.

Acknowledgements

This work was supported by grants from the Swedish Society of Medicine and the Swedish Medical Research Council (project no. K98-17X-12650). The authors thank Jan-Olof Andersson for preparing PRAP-1.

References

1 Menzies D, Ellis H. Intestinal obstruction from adhesions – how big is the problem? *Ann R Coll Engl* 1990; **72**: 60–3.
2 Menzies D. Postoperative adhesions: their treatment and relevance in clinical practice. *Ann R Coll Surg Engl* 1993; **75**: 147–53.

3 Diamond MP, Hershlag A. Adhesion formation/reformation. *Prog Clin Biol Res* 1990; **358:** 23–33.
4 Van Der Krabben AA, Dijkstra FR, Nieuwenhuijzen M, Reijnen MM, Schaapveld M, Van Goor H. Morbidity and mortality of inadvertent enterotomy during adhesiotomy. *Br J Surg* 2000; **87:** 467–71.
5 Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D *et al.* Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. *Lancet* 1999; **353:** 1476–80.
6 Holmdahl L. Making and covering of surgical footprints. *Lancet* 1999; **353:** 1456–7.
7 Raftery AT. Effect of peritoneal trauma on peritoneal fibrinolytic activity and intraperitoneal adhesion formation. An experimental study in the rat. *Eur Surg Res* 1981; **13:** 397–401.
8 Holmdahl L, Eriksson E, al-Jabreen M, Risberg B. Fibrinolysis in human peritoneum during operation. *Surgery* 1996; **119:** 701–5.
9 Ivarsson M-L, Holmdahl L, Eriksson E, Söderberg R, Risberg B. Expression and kinetics of fibrinolytic components in plasma and peritoneum during abdominal surgery. *Fibrinolysis Proteolysis* 1998; **12:** 61–7.
10 Ivarsson M-L, Bergström M, Eriksson E, Risberg B, Holmdahl L. Tissue markers as predictors of postoperative adhesions. *Br J Surg* 1998; **85:** 1549–54.
11 Abrahamsson T, Björquist P, Deinum J, Ehnebom J, Hulander M, Legnehed A *et al.* An antibody fragment against PAI-1 inhibits PAI-1 activity and stimulates fibrinolysis *in vitro* and *in vivo*. *Fibrinolysis* 1994; **8**(Suppl 2): 55–6.
12 Abrahamsson T, Nerme V, Strömqvist M, Åkerblom B, Legnehed A, Pettersson K *et al.* Anti-thrombotic effect of a PAI-1 inhibitor in rats given endotoxin. *Thromb Haemost* 1996; **75:** 118–26.
13 Holmdahl L, al-Jabreen M, Risberg B. Experimental models for quantitative studies on adhesion formation in rats and rabbits. *Eur Surg Res* 1994; **26:** 248–56.
14 Holmdahl LE, Al-Jabreen M, Risberg B. Role of fibrinolysis in the formation of postoperative adhesions. *Wound Repair Regen* 1994; **2:** 171–6.
15 Falk K, Holmdahl L, Halvarsson M, Larsson K, Lindman B, Bengmark S. Polymers that reduce intraperitoneal adhesion formation. *Br J Surg* 1998; **85:** 1153–6.
16 van Giezen JJ, Wahlund G, Nerme V, Abrahamsson T. The Fab-fragment of a PAI-1 inhibiting antibody reduces thrombus size and restores blood flow in a rat model of arterial thrombosis. *Thromb Haemost* 1997; **77:** 964–9.
17 Holmdahl L, Eriksson E, Rippe B, Risberg B. Kinetics of transperitoneal tissue-type plasminogen activator absorption. *Fibrinolysis* 1996; **10:** 1–7.

Exhibit D

Matrix metalloproteinases in repair

WILLIAM C. PARKS, PhD

During repair, many different matrix metalloproteinases are produced by multiple cell types residing in various compartments within the wound environment. This diversity of enzymes, coupled with discreet cellular expression, implies that different matrix metalloproteinases serve different functions, acting on a variety of substrates, during wound healing. With few exceptions, however, the actual function and spectrum of functions of matrix metalloproteinases in vivo is not known. Even with the advent of genetically defined animal models, few studies have rigorously addressed the substrates and role of matrix metalloproteinases in wound repair. Before we can understand the role of matrix metalloproteinases in ulceration and disease, we need to determine the function these enzymes serve in normal tissues and repair.
(WOUND REP REG 1999;7:423–432)

The matrix metalloproteinases (MMPs) comprise a family of enzymes which share several common properties.[1] MMPs, or matrixins, are a subgroup of the much larger metalloproteinase superfamily, which also includes astacin and ADAM proteinases, among others. To date, greater than 20 different MMPs have been characterized, and additional members continue to be identified. To be classified as a *matrix* metalloproteinase, a protein must have the following five features. First, as for all metalloproteinases, its catalytic mechanism must require an active site Zn^{2+} that binds a conserved histidine-containing domain, HEXXHXXGXXH. Second, its prodomain should be about 80 amino acids and contain the consensus sequence PRCXXPD. In an inactive state, the conserved cysteine residue in the prodomain provides the fourth coordination site for the catalytic zinc ion. Third, a putative MMP must be synthesized as inactive zymogen. Most MMPs are secreted or, as for the membrane-type MMPs (MT-MMP), are integrated in membranes in an inactive state. Fourth, a would-be MMP must be able to degrade or cleave at least one extracellular matrix (ECM) protein. Actually, most MMPs can degrade several matrix proteins. Finally, its proteolytic activity should be inhibited to some degree by tissue inhibitors of metalloproteinases (TIMPs), of which there are currently four. Detailed information of the MMP family and individual members can be found in recent books and reviews.[2–5]

ECM	Extracellular matrix
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type MMP
TIMP	Tissue inhibitor of metalloproteinase

Typically MMPs are not expressed in normal, healthy, resting tissues, at least their production and activity, with notable exceptions, are maintained at nearly undetectable levels. In contrast, some level of MMP expression is seen in any repair or remodeling process, in any diseased or inflamed tissue, and in essentially any cell type grown in culture. Although the qualitative pattern and quantitative levels of MMPs varies among tissues, diseases, tumors, inflammatory conditions, and cell lines, a reasonably safe generalization is that activated cells express MMPs. This is neither a controversial or surprising conclusion. After

From the Departments of Pediatrics (Allergy and Pulmonary Medicine) and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri
Reprint requests: William C. Parks, PhD, Department of Pediatrics, Box 8116, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Fax: (314) 454-5372; Email: parks_w@kids.wustl.edu.

ISSN: 1067-1927 $5.00 + 0

Table 1. Distribution of matrix metalloproteinases among cell types during repair of cutaneous wounds

		Cell type		
Enzyme	**MMP number**	**Keratinocytes**	**Dermal cells**	**Inflammatory cells**
Collagenase-1	MMP-1	+	+	+
Collagenase-2	MMP-8	–	–	+
Collagenase-3	MMP-13	–	+	–
Stromelysin-1	MMP-3	+	+	–
Stromelysin-2	MMP-10	+	–	–
Gelatinase-A	MMP-2	–	+	–
Gelatinase-B	MMP-9	+	–	+
Matrilysin	MMP-7	–	–	–
Macrophage metalloelastase	MMP-12	–	–	+
MT1-MMP	MMP-14	–	+	–

all, matrix is remodeled in situations associated with disease or tissue activation, such as inflammation, cancer, and normal resorption and repair, and MMPs participate in matrix turnover, or at least we think they do. In this review, I shall focus on the potential and documented functions of MMPs in repair of cutaneous wounds. As essentially all known MMPs are expressed by several different cell types at some time during wound repair (Table 1), this is an appropriate model to study and discuss proteinase biology.

Before we can understand the function of MMPs, we need to know where, when, and by which cells these proteinases are produced. By knowing the temporal and spatial patterns of MMP expression, we can then formulate cogent hypotheses to address how these enzymes function in precise tissue compartments at distinct stages of repair. Even with this important information, understanding the role of a specific proteinase is not straightforward for two main reasons. First, as stated, most, if not all, MMPs are expressed in response to injury, thus potentially confounding deciphering the role of any specific enzyme. Second, and more important, the actual in vivo function and scope of functions of a given MMP are not known. I elaborate upon these two points below.

MANY SUBSTRATES, MANY MMPS

ECM is composed of several different proteins providing multiple functions. Some proteins, such as elastin and many collagen types, are deposited as large, insoluble, protease-resistant fibers and confer strength and resiliency to tissues. Several different matrix glycoproteins, such as laminins, fibronectin, thrombospondins, fibrillins, entactin, and many others, form aggregates of varying degrees of complexity, providing a substratum for cell adhesion, and are important for numerous protein–protein interactions. Proteoglycans, in which the mass of carbohydrate exceeds the mass of protein, function in tissue hydration. In addition, extracellular molecules can be organized into specialized structures, such as ligaments, bone, and basement membrane, the matrix upon which epithelia and endothelial cells reside. Essentially all matrix proteins have some role in affecting cell phenotype and behavior, and relevant to this review, all can be degraded by MMPs.

Although each MMP is often described as having a specific substrate specificity, individual enzymes can act on many different proteins. In addition, and more important, the spectrum of substrates among enzymes is actually quite similar. For example, gelatinase-A (MMP-2), gelatinase-B (MMP-9), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), matrilysin (MMP-7), macrophage metalloelastase (MMP-12), and collagenase-3 (MMP-13) can each degrade many of the same matrix substrates, such as denatured and native nonfibrillar collagens, elastin, basement membrane components, fibrillins, fibronectin, and more. Distinct from other MMPs, collagenase-1 (MMP-1) and collagenase-2 (MMP-8, neutrophil collagenase) seem to have a very defined substrate spectrum, being limited to the fibrillar collagens, types I, II, and III. In addition, collagenase-1 and -2 do not degrade collagen, but rather make a single, site-specific cleavage within the triple helix of these abundant matrix components.[6] Once cleaved, the triple helix of collagen fibrils relaxes at body temperature, and the partially unwound fibril becomes susceptible to further proteolysis by a variety of other MMPs and proteinases of other families. Aside from the collagenases, however, the substrates that

overlap among MMPs is more impressive than the list of selective substrates.

In a setting like a repairing wound, in which essentially all MMPs are present, the shared substrate specificity among enzymes would permit biochemical redundancy. However, substrate selectivity can be honed by two processes: enzyme affinity and compartmentalization. Kinetic studies have shown that specific enzymes degrade some substrates more efficiently than others. For example, the gelatinases (MMP-2, MMP-9) act on cleaved collagen better than other MMPs;[7] matrilysin (MMP-7) is a more potent proteoglycanse than is stromelysin-1 (MMP-3) or gelatinase-B (MMP-9);[8] macrophage metalloelastase (MMP-12) is the most elastolytic enzyme of the MMP family;[9] and as stated, only the collagenases (MMP-1, -8, and -13) can cleave native fibrillar collagens. Thus, in a complex tissue environment in which many types and forms of matrix proteins are present, selectivity of MMP catalysis may be directed, in part, by the concentration of a preferred substrate relative to that of other potential substrates deposited within the range of a secreted MMP.

COMPARTMENTALIZATION: PUT THE PROTEASE WHERE IT IS NEEDED

MMPs are not actively expressed in uninjured skin, either in the epidermis or dermis (Figure 1). Gelatinase-A (MMP-2) may be an exception to this rule. Though its mRNA is not detectable in normal skin,[10,11] the enzyme protein is detected by zymographic analysis.[12] Such observations have led to the idea that gelatinase-A is stored in the matrix awaiting activa-

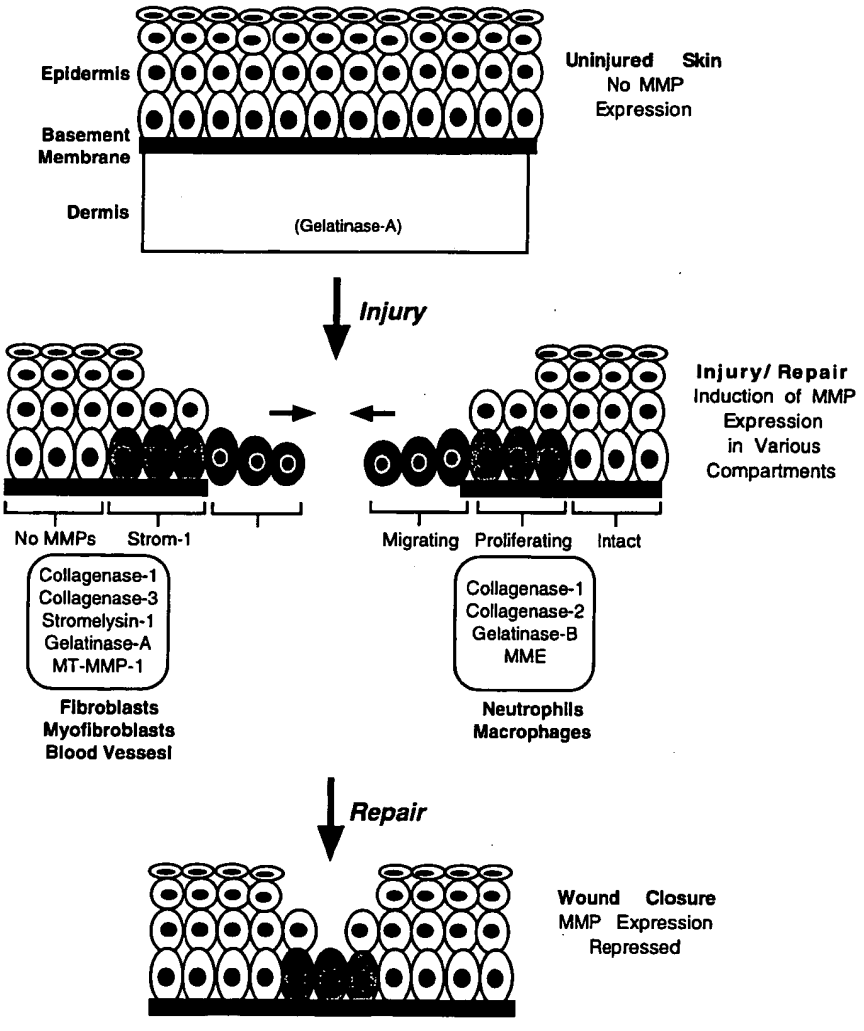


Figure 1. Compartmentalization of MMP expression in the epidermis and dermis during wound healing. In uninjured, healthy skin, expression of MMPs is not detected. (An exception is the active production of matrilysin in skin glands.) Low levels of gelatinase-A protein can be extracted from skin, but its mRNA is at undetectable levels. In response to injury, several MMPs are expressed by defined cells in specific locations. Collagenase-1, stromelysin-2, and gelatinase-B are invariably expressed by migrating basal keratinocytes which have moved off of the basement membrane (*black line*). Stromelysin-2 is also expressed in the epidermis but by a functionally distinct population of basal keratinocytes. In the dermis, mesenchymal and inflammatory cells produce mostly distinct spectra of MMPs. At the completion of re-epithelialization, MMP expression is turned off.

tion by migrating cells.[13,14] Another exception is matrilysin (MMP-7), which is actively expressed by the ductal epithelium in all sweat and eccrine glands;[15] however, this enzyme does not seem to have a role in repair of cutaneous wounds (Table 1). Aside from these two exceptions, the MMPs that are prominently expressed in injured skin are not present, at least to any significant level, in normal skin.

By which cells and where in the tissue environment an MMP is expressed and released are equally, if not more important considerations when predicting the target of proteolysis than is the affinity of enzyme–substrate interactions. After all, cells do not release proteases indiscriminately, especially enzymes like collagenases with such a defined substrate specificity, but rather they rely on precise cell : matrix interactions to accurately remodel connective tissue in the pericellular space. In addition, cell-matrix contacts can provide an unambiguous signal informing the cell which matrix protein it has encountered and, hence, which proteinase is needed and where it should be delivered and released. For example, in human wounds, collagenase-1 is induced in basal keratinocytes as the cells move off of the basement membrane and contact type I collagen in the underlying dermis (Figure 1).[16] This response is specifically controlled by the collagen-binding integrin $\alpha_2\beta_1$,[17] which also directs secretion of collagenase-1 to the points of cell-matrix contact (unpublished data). Furthermore, only basal keratinocytes in contact with dermal type I collagen at the migrating front express collagenase-1. Cells migrating across an intact basement membrane, as seen in some forms of blisters, do not express collagenase-1.[16,18] Expression of collagenase-1 is rapidly turned off once re-epithelialization is complete and the basement membrane begins to reform.[19] This example demonstrates that expression and activity of a specific MMP can be confined to a specific location in the wound (the superficial plane of the open wound bed) and to a specific stage of repair (re-epithelialization). As discussed below, collagenase-1 predictably has a role in cell migration.

EPIDERMAL MMPS

Similar to collagenase-1, stromelysin-2 and gelatinase-B are also expressed by basal keratinocytes at the wound edge (Figure 1).[11,20,21] Although contact with dermal type I collagen induces collagenase-1, the signals controlling the site-specific release of stromelysin-2 and gelatinase-B have not been identified. Stromelysin-1 (MMP-3) is also produced by basal keratinocytes but by a functionally distinct subpopulation of cells.[20] Whereas collagenase-1, stromelysin-2, and gelatinase-B are released by cells at the forward edge of migration, stromelysin-1 is expressed by keratinocytes just behind this group in a hyperproliferative population of basal keratinocytes (Figure 1).

Although the location of stromelysin-1, stromelysin-2, and gelatinase-B in the wounded epidermis imply that these MMPs function in proliferation and migration, the role of these enzymes have not yet been determined. Complicating this issue is that stromelysin-1 and gelatinase-B deficient mice do not show a noticeable delay or change in wound closure,[22–24] though these mice reveal many other phenotypes when challenged (Table 2). Forced over-expression of stromelysin-1 in mammary epithelial cells mediates a

Table 2. Phenotype of MMP knockout mice

		Phenotype	
gene	**Lethal**	**Unchallenged mice (ref)**	**Wounding and other related models (ref)**
Stromelysin-1	No	None[22]	Impaired wound contraction[23,24] Reduced contact hypersensitivity response[56] Accelerated arthritis[22]
Gelatinase-A	No	None[57]	Reduction in angiogenesis and tumor growth[58]
Gelatinase-B	No	Transient slowing of long bone growth secondary to reduced angiogenesis[51]	Normal neutrophil extravasation[59] Resistant to induced blister formation[59] Persistent contact hypersensitivity response[56] Reduced metastasis[60]
Matrilysin	No	Lack of activated antimicrobial peptides[52]	Inability to repair mucosal epithelial wounds[55] Reduced ability to kill pathogenic bacteria[52] Reduced tumorigenesis[61]
Macrophage metalloelastase	No	None[39]	Reduced elastolytic capacity of macrophages[39] Reduced ability of macrophages to migrate through matrix[39] Protection against smoking-induced emphysema[62]
MT1-MMP	Yes	Severe skeletal abnormalities[50]	Not yet assessed

transition to a migratory phenotype, possibly by cleaving the ectodomain of E-cadherin in desmosomes.[25] Although desmosomes are dissembled in keratinocytes at the wound edge, this process, which is necessary for efficient migration, does not correlate spatially with the expression of stromelysin-1, but it does with that for stromelysin-2 (Figure 1). Interestingly, stromelysin-2 is also produced by mucosal cells at the edge of gut and lung injuries.[26] Hence, this MMP is produced by migrating cells in diverse forms of epithelia and, because it has been hardly seen elsewhere in human tissue, stromelysin-2 may be an injury-specific protease. As it can degrade the same proteins as does stromelysin-1, stromelysin-2 may be involved in desmosome disassembly during migration, but more specific models will be needed to test this idea.

DERMAL MMPS

In the underlying wound bed, a variety of MMPs are expressed by resident and infiltrating cells. Fibroblasts, myofibroblasts, endothelial cells, and perivascular cells throughout the wound bed express gelatinase-A, MT1-MMP, stromelysin-1, collagenase-1, and collagenase-3 during all stages of repair[10,11,27,28] (Table 1 and Figure 1). Infiltrating cells, particularly neutrophils and macrophages, contribute MMPs, as well. Neutrophils store and release collagenase-2 (MMP-8) and large quantities of gelatinase-B, but these cells lack the capacity to produce more enzyme in the tissue space.[29,30] In fact, it is likely that most of the gelatinase-B detected in wound fluid, which is one of the more prominent proteinases in this compartment,[31] is derived from neutrophils. Although macrophages can express a variety of MMPs,[32] they seem to produce a limited number of these enzymes in cutaneous wounds. In addition, the enzymes expressed by macrophages differ at distinct stages of repair. Early in wound repair, they produce collagenase-1, but at later stages, several days after re-epithelialization is complete, they begin to express macrophage metalloelastase (MMP-12).[28,33] Thus, a variety of MMPs are produced by different cells in different locations at different times.

FUNCTION OF MMPS IN REPAIR

Based on the discussion above, it follows that the distinct spatial and temporal patterns of MMPs in wounds indicate that different MMPs serve distinct functions during repair. In addition, enzymes that are present in both the epidermis and dermis, like collagenase-1 (Figure 1), likely serve different functions in different compartments. As stated, the actual substrate and, consequently, the function of most MMPs is not known, and as such, the function of metalloproteinases is usually more presumed than proved. These enzymes are called *matrix* metalloproteinases because they *can* degrade matrix molecules. This designation arose largely from numerous studies showing that purified matrix proteins are degraded by purified, activated MMPs when combined under optimal conditions, but few examples exist documenting that matrix degradation in a tissue environment is caused *directly* by the catalytic activity of a metalloproteinase. The ability of MMPs to act on matrix proteins does not preclude activities beyond connective tissue turnover or destruction.

Most studies provide correlative or associative data, though often quite convincing of a role in matrix turnover, yet stop short of providing a firm and direct causative link between MMP activity and protein degradation. For example, over expression of stromelysin-1 in breast epithelium leads to selective degradation of entactin/nidogen in the basement membrane, which is rescued by expression of TIMP-1,[34] and macrophage metalloelastase is required for macrophage-mediated elastolysis and tissue damage (Table 2). Although these elegant studies relied on defined genetic approaches and represent state-of-the-art work in proteinase biology, the findings do not prove that the MMP in question acts directly on the targeted matrix protein or if matrix degradation is mediated via an another enzyme whose activity is dependent on the metalloproteinase. The concerns raised here are not meant to diminish the findings contributed by any group, but rather are meant to underscore the difficulty in determining the precise mechanistic function of a metalloproteinase in a complex environment.

Because of the restricted substrate specificity of collagenase-1 and collagenase-2, the presence of these enzymes likely do indicate that fibrillar collagen is being cleaved. Indeed, excess collagen is evident in tissues of mice that produce collagenase-resistant type I collagen,[35] and a neoepitope revealed by collagenase cleavage of type II collagen is evident in osteoarthritic cartilage.[36] These studies, in addition to others, show that some MMPs, at least collagenases, do act on matrix substrates in vivo. However, the mere presence of an MMP in a tissue sample, either normal, inflamed, or diseased, has led investigators to assume that the proteinases identified are involved in remodeling the local extracellular matrix. Because most MMPs can degrade a wide range of structurally diverse connective tissue molecules, it is likely that this class of proteinases can act on many proteins. Thus, as we con-

sider the potential functions and consequences of metalloproteinase activity in wound repair, we would should not limit our thinking to matrix degradation.

COLLAGENASE-1 AND MIGRATION

Of the many MMPs expressed throughout wounds, the function of collagenase-1 in the migrating epidermis is the best understood, and this function requires the ability of this proteinase to act on matrix. The invariable expression of collagenase-1 by basal keratinocytes in all forms of wounds and the confinement of its expression to periods of active re-epithelialization indicates that this enzyme participates in cell migration. Beyond directly remodeling structural proteins, such as during morphogenesis and tissue resorption, MMPs are thought to breakdown matrix barriers that impede cell migration. Clearly, this is a reasonable role for these proteinases in facilitating cell movement through a three-dimensional matrix, as is seen during blastocyst invasion,[37] angiogenesis,[38] and extravasation and infiltration of inflammatory cells.[39] During normal re-epithelialization, however, keratinocytes migrate along a path of least resistance, dissecting underneath the scab while remaining superficial to the underlying viable dermis and wound bed.[40] Thus, epidermal repair involves cell migration in a two-dimensional plane rather than through a three-dimensional matrix-rich environment. Findings from our group indicate that collagenase-1 is a component of a molecular machine that drives and orients keratinocyte migration.[17] Other key components of this machine are native type I collagen in the dermis and the integrin $\alpha_2\beta_1$ on keratinocytes.

The requirement of collagenase-1 for keratinocyte movement was documented in various migration assays. In all assays, keratinocyte migration on native collagen was completely blocked by treatment with a broad-acting peptide hydroxamate inhibitor. This compound, similar to many MMP inhibitors, is a substrate-based inhibitor containing a hydroxamic acid moiety which chelates the active site zinc cation and renders MMPs catalytically inactive.[41] Because these early generation hydroxamate compounds inhibit all MMPs and because keratinocytes express other MMPs (Figure 1), these experiments do not show that the activity of collagenase-1 is required for keratinocyte migration on collagen. However, keratinocytes plated on collagenase-resistant mutant collagen do not migrate, yet the cells express collagenase-1 and adhere equally to those on wild-type collagen.[17] This collagen matrix was isolated from mice in which a double mutation was inserted by homologous recombination in the region of the collagenase cleavage site of the α1(I) chain.[35,42]

Because all three human collagenases can make the same cleavage in type I collagen, the experiment with the mutant substrate did not show that collagenase-1 is specifically required for keratinocyte migration on a collagen matrix. Reagents that selectively block the activity of collagenase-1 verified that this MMP alone is necessary and sufficient for keratinocyte migration on a collagen-containing matrix. Keratinocyte migration is completely inhibited by affinity-purified anticollagenase-1 antibodies, which block the enzyme's catalytic activity.[17] Furthermore, treatment with a newer generation hydroxymate compound, which inhibits all MMPs *except collagenase-1*, does not affect keratinocyte migration (unpublished observations). Together, these data show that the proteolytic activity of collagenase-1, and not that of any other MMP, is required for keratinocyte migration on native type I collagen.

COLLAGENASE-1 FACILITATES CELL DIRECTIONALITY

Based on the above observations, we proposed that collagenase-1 acting on its principal substrate in the dermis, type I collagen, provides migrating keratinocytes with a mechanism to maintain their course and directionality in the wound environment during re-epithelialization. Basal keratinocytes constitutively express the type I collagen-binding integrin $\alpha_2\beta_1$ on their basolateral surfaces. In wounds, $\alpha_2\beta_1$ becomes concentrated at the forward-basal tip of migrating keratinocyte.[43–45] This redistribution, which is likely regulated by an initial contact with dermal collagen, places $\alpha_2\beta_1$ in intimate contact with dermal type I collagen (Figure 2). Contact with collagen induces collagenase-1, and this expression is mediated by $\alpha_2\beta_1$.[17]

Because $\alpha_2\beta_1$ binds native collagen with high affinity,[46] clustering this integrin at contact points would tether keratinocytes to the dermis rendering them unable to migrate. Collagenase-1 aids in dissociating keratinocytes from these high affinity attachments by altering the nature of the collagen matrix and, in turn, its affinity with $\alpha_2\beta_1$. As stated, collagenase-1 makes a single, site-specific cleavage through the triple helix about 3/4 the length from the N-terminus. The resultant TC^A and TC^B fragments are thermally unstable at body temperature and spontaneously unwind into gelatin,[6] which binds $\alpha_2\beta_1$ with

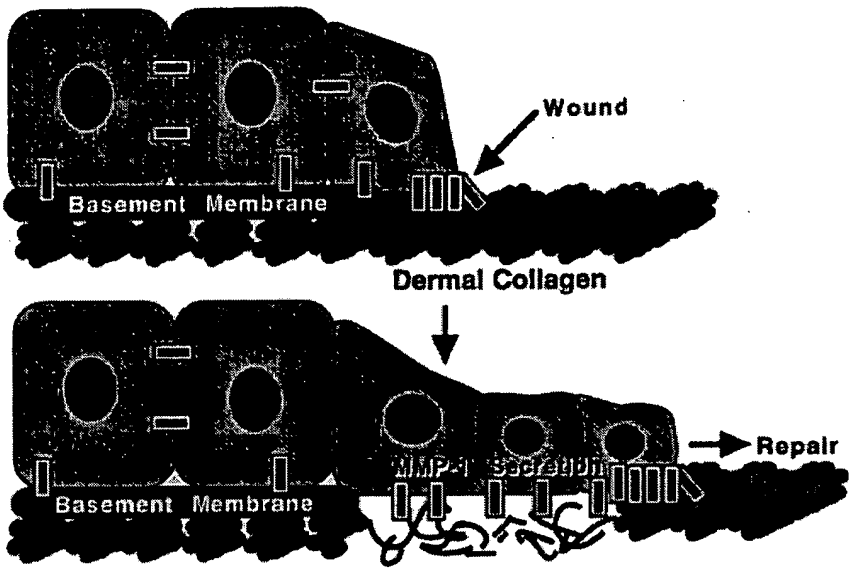


Figure 2 Collagenase-1-dependent keratinocyte migration during epidermal wound repair. After wounding, basal keratinocytes dislodge from the basement membrane and contact dermal type I collagen. The collagen-binding integrin $\alpha_2\beta_1$ ([]) accumulates at the frontobasal surface of migrating keratinocytes. $\alpha_2\beta_1$ binds dermal collagen with high affinity, and this interaction induces collagenase-1 expression. High affinity binding to collagen hinders cell motility. Cleavage of type I collagen by collagenase-1, and its subsequent conversion to gelatin, loosens the $\alpha_2\beta_1$ integrins hold with the matrix, thereby allowing the keratinocytes to migrate. The high-affinity interaction of $\alpha_2\beta_1$ with dermal collagen, but not with gelatin, provides the migrating cells with a mechanism to control their direction and to remain superficial during re-epithelialization.

a much lower affinity than does native collagen.[46] Thus, by simply making a single cut through the type I collagen helix, collagenase-1 effectively mediates the loosening of the tight contacts keratinocytes establish with the dermal matrix, thereby allowing the cells to move forward. This function is distinct from the often suggested idea that migrating cells use MMPs to remove matrix barriers that may physically impede movement.

Although collagenase-1 facilitates keratinocyte migration by affecting the conformation of type I collagen and, consequently, the avidity with which cells interact with it, one may argue that this is an inherently inefficient mechanism. If activated keratinocytes migrate *over* the viable dermis, rather than *through* matrix, then why do they need to cleave type I collagen? Why would they adhere to the dermis with such high affinity if their objective is to close the wound as quickly as possible? The answer, we believe, is that the process of interacting with and then cleaving type I collagen provides keratinocytes with a mechanism to determine and maintain their directionality during re-epithelialization. Because $\alpha_2\beta_1$ binds native collagen much more tightly than it binds gelatin, this integrin, once loosened by the action of collagenase-1, would favor re-establishment of high affinity interactions with native, uncleaved collagen. Thus, by repeatedly establishing tight contacts, then rapidly loosening this hold by the action of collagenase-1, keratinocytes use native type I collagen as a "molecular compass" guiding repair over the open wound surface.

An important observation relevant to the directionality hypothesis is that collagenase-1 production is induced in keratinocytes by native type I collagen but not by denatured forms of the molecule.[47,48] Thus, collagenase-1 acting on collagen creates a mediator that does not support or maintain its own production. The conversion of collagen to gelatin would replace the inductive stimulus with a neutral substrate (gelatin) and, in stationary cells, collagenase-1 expression would decline. Indeed, collagenase-1 expression is rapidly turned off at the completion of re-epithelialization.[19] The initial expression of collagenase-1 and cleavage of the collagen substrate would, in effect, neutralize the inductive effect of the underlying matrix. If keratinocytes continue to interact with type I collagen, presumably by migrating, then they would continue to express collagenase-1, which they do throughout re-epithelialization.

LESSONS FROM KNOCK-OUTS

To date, six MMPs have been knocked out in mice by targeted mutagenesis (Table 2). With the exception of MT1-MMP-deficient mice, no other MMP knock outs show an overt phenotype related to connective tissue production or turnover in the unchallenged animal. This lack of matrix phenotype may indicate biochemical redundancy among MMPs; remove one, and

the others fill in. Indeed, stromelysin-1 is over-expressed in the uterus of matrilysin-deficient mice, and matrilysin is over-expressed in stromelysin-1-deficient mice.[49] However, in the mouse uterus, matrilysin is expressed by the epithelium and stromelysin-1 is produced by interstitial cells, and thus, it is not clear how over-expression of an enzyme in one compartment can compensate for the activity of another proteinase in a separate, distant area. Alternatively, the lack of a matrix phenotype may indicate that other classes of proteinases are responsible for matrix turnover or that the MMPs involved in normal matrix remodeling have not yet been knocked out. Thus, it was comforting to see the recent data on the MT1-MMP-null mouse, which has a marked defect in its ability to turnover collagen.[50]

Most MMP-null mice show no or only a minor phenotype in the unchallenged animal (Table 2). Gelatinase-B-deficient mice show a postnatal delay in long bone growth, but this defect is transient with no lingering phenotype in the mature animal.[51] Matrilysin-deficient mice do not activate intestinal antimicrobial peptides, but unchallenged mice, which are housed in pristine barrier facilities, are not susceptible to bacterial infections.[52] In contrast, MMP knock out mice show several phenotypes in models of injury, infection, inflammation, and induced tumorigenesis (Table 2); however, few defects or changes in cutaneous wound repair have been noted. A notable exception are stromelysin-1-deficient mice in which wound contraction is impaired.[23,24] Although the mechanism of how stromelysin-1 functions in contraction is not known, this phenotype is consistent with the expression of this MMP by dermal fibroblasts in a variety of wounds.[11,20,28,53] Re-epithelialization of skin wounds is not impaired in stromelysin-1 and gelatinase-B-deficient mice, even though these MMPs are expressed by wound edge keratinocytes (Figure 1).

Matrilysin-deficient mice have shown the most severe wound-repair defect among MMP knock out mice. Unlike most MMPs, matrilysin (MMP-7) is not found in cutaneous wounds (Table 1), but it is expressed by migrating epithelial cells in wounds and ulcerations of mucosal tissues, such as lung and intestine.[54,55] Showing an essential role for matrilysin in mucosal repair, airway[55] or colonic (unpublished data) epithelial wounds do not repair in MMP-7-null mice. Even several days after injury, the epithelium at the wound edge shows no sign of migration; however, the role of matrilysin in cell movement is not known. The pattern of matrilysin expression in mucosal wounds parallels that of collagenase-1 in cutaneous wounds, and collagenase-1 is not produced by mucosal epithelium in vivo.[54,55] In addition to the programmed responses of differentiated cells, the composition of the underlying interstitial matrix may dictate the pattern of MMP expression between skin and mucosa. Thus, whereas collagenase-1 facilitates migration of keratinocytes over the collagen-rich matrix of dermis, matrilysin would be a more appropriate proteinase to remodel matrix components of the lamina propria, which include elastin, adhesive glycoproteins, and proteoglycans. The basally directed secretion of matrilysin during migration[55] supports the idea that this epithelial MMP acts on matrix proteins during repair.

CONCLUSION

In this article, I have focused the discussion on the role and pattern of MMPs in normally healing wounds. However, several studies have shown that excessive levels of many MMPs are present in ulcerations and in chronically inflamed tissues throughout the body. These observations have led to oft held concept that excessive proteolysis damages tissues and impairs healing. Indeed, unregulated, excessive proteolysis must be responsible for the destruction of matrix proteins in arthritis, aneurysms, and other conditions of structural tissues, but not all proteinases expressed in diseased tissue necessarily contribute to disease pathogenesis. For example, matrilysin is markedly over-expressed in lungs of patients with cystic fibrosis,[55] a severe disease characterized by mucosal injury, interstitial fibrosis, inflammation, and infection. Because matrilysin can vigorously degrade all sorts of matrix proteins, it may contribute to lung damage. Based on the mechanisms uncovered in matrilysin-null mice,[52,55] over-expression of this MMP may reflect its beneficial roles in epithelial cell migration and in mucosal defense against bacteria. Because MMPs can both break down matrix and process secreted precursor proteins, we cannot conclude by its presence alone if a specific proteinase in an inflammatory or injury setting is contributing to a reparative or disease process.

REFERENCES

1. Woessner JF Jr. The matrix metalloproteinase family. In: Parks WC, Mecham RP, editors. Matrix metalloproteinases. San Diego: Academic Press, 1998: 1–14.
2. Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993;4:197–250.

3. Kähäri VM, Saarialho-Kere U. Matrix metalloproteinases in skin. Exp Dermatol 1997;6:199–213.
4. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998;10:602–8.
5. Parks WC, Mecham RP. Matrix metalloproteinases San Diego: Academic Press, 1998.
6. Jeffrey JJ. Interstitial collagenases. In: Parks WC, Mecham RP, editors. Matrix metalloproteinases. San Diego: Academic Press, 1998: 15–42.
7. Mackay AR, Hartzler JL, Pelina MD, Thorgeirsson UP. Studies on the ability of 65-kDa and 92-kDa tumor cell gelatinases to degrade type IV collagen. J Biol Chem 1990;265:21929–34.
8. Halpert I, Roby JD, Sires UI, Potter-Perigo S, Wight TN, Welgus HG, Shapiro SD, Wickline SA, Parks WC. Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc Natl Acad Sci USA 1996;93:9748–53.
9. Shapiro SD. Elastolytic metalloproteinases produced by human mononuclear phagocytes. Potential roles in destructive lung disease. Am J Respir Crit Care Med 1994;150:S160–4.
10. Stricklin GP, Li L, Nanney LB. Localization of mRNAs representing interstitial collagenase, 72-kDa gelatinase, and TIMP in healing porcine burn wounds. J Invest Dermatol 1994;103:352–8.
11. Okada A, Tomasetto C, Lutz Y, Bellocq J-P, Rio M-C, Bassett P. Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. J Cell Biol 1997;137:67–78.
12. Agren MS. Gelatinase activity during wound healing. Br J Dermatol 1994;131:634–40.
13. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. Cell 1997;91:439–42.
14. Murphy G, Gavrilovic J. Proteolysis and cell migration: creating a path? Curr Opin Cell Biol 1999;11:614–21.
15. Saarialho-Kere UK, Crouch EC, Parks WC. Matrix metalloproteinase matrilysin is constitutively expressed in human exocrine epithelium. J Invest Dermatol 1995;105:190–6.
16. Saarialho-Kere UK, Kovacs SO, Pentland AP, Olerud J, Welgus HG, Parks WC. Cell–matrix interactions modulate interstitial collagenase expression by human keratinocytes actively involved in wound healing. J Clin Invest 1993;92:2858–66.
17. Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J Cell Biol 1997;137:1445–57.
18. Saarialho-Kere UK, Vaalamo M, Airola K, Niemi K-M, Oikarinen AI, Parks WC. Interstitial collagenase is expressed by keratinocytes which are actively involved in re-epithelialization in blistering skin diseases. J Invest Dermatol 1995;104:982–8.
19. Inoue M, Kratz G, Haegerstrand A, Stahle-Bäckdahl M. Collagenase expression is rapidly induced in wound-edge keratinocytes after acute injury in human skin, persists during healing, and stops at re-epithelialization. J Invest Dermatol 1995;104:479–83.
20. Saarialho-Kere UK, Kovacs SO, Pentland AP, Parks WC, Welgus HG. Distinct populations of keratinocytes express stromelysin-1 and -2 in chronic wounds. J Clin Invest 1994;94:79–88.
21. Madlener M, Mauch C, Conca W, Brauchle M, Parks WC, Werner S. Growth factor regulation of stromelysin-2 expression by keratinocytes: implications for normal and impaired wound healing. Biochem J 1996;320:659–64.
22. Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, McDonnell J, Singer II, Bayne EK, Flanagan J, Kawka D, Shen CF, Stevens K, Chen H, Trumbauer M, Visco DM. Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction. Arthritis Rheum 1998;41:110–21.
23. Bullard KM, Lund L, Mudgett JS, Mellin TN, Hunt TK, Murphy B, Ronan J, Werb Z, Banda MJ. Impaired wound contraction in stromelysin-1-deficient mice. Ann Surg 1999;230:260–5.
24. Bullard KM, Mudgett J, Scheuenstuhl H, Hunt TK, Banda MJ. Stromelysin-1-deficient fibroblasts display impaired contraction in vitro. J Surg Res 1999;84:31–4.
25. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol 1997;139:1861–72.
26. Vaalamo M, Karjalainen-Lindsberg ML, Puolakkainen P, Kere J, Saarialho-Kere U. Distinct expression profiles of stromelysin-2 (MMP-10), collagenase-3 (MMP-13), macrophage metalloelastase (MMP-12), and tissue inhibitor of metalloproteinases-3 (TIMP-3) in intestinal ulcerations. Am J Pathol 1998; 152:1005–14.
27. Stricklin GP, Nanney LB. Immunolocalization of collagenase and TIMP in healing human burn wounds. J Invest Dermatol 1994;103:488–92.
28. Madlener M, Parks WC, Werner S. Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially regulated during excisional skin wound repair in mice. Exp Cell Res 1998;242:201–11.
29. Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM, Mainardi CL. Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. J Biol Chem 1990; 265:11421–4.
30. Stahle Bäckdahl M, Parks WC. 92 kDa gelatinase is actively expressed by eosinophils and secreted by neutrophils in invasive squamous cell carcinoma. Am J Pathol 1993;142:995–1000.
31. Wysocki AB, Staiano-Coico L, Grinnell F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. J Invest Dermatol 1993;101:64–8.
32. Welgus HG, Senior RM, Parks WC, Kahn AJ, Ley TJ, Shapiro SD, Campbell EJ. Neutral metalloproteinases produced by human mononuclear phagocytes: a prominent role of cellular differentiation. Matrix 1992;Suppl. 1:363–7.
33. Saarialho-Kere UK, Chang ES, Welgus HG, Parks WC. Distinct localization of collagenase and TIMP expression in wound healing associated with ulcerative pyogenic granuloma. J Clin Invest 1992;90:1952–7.
34. Alexander CM, Howard EW, Bissell MJ, Werb Z. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. J Cell Biol 1996;135:1669–77.
35. Liu X, Wu H, Byrne M, Jeffrey JJ, Krane S, Jaenisch R. A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. J Cell Biol 1995;130:227–37.
36. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534–45.
37. Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH, Fisher SJ. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J Cell Biol 1991;113:437–49.
38. Fisher C, Gilbertson-Beadling S, Powers EA, Petzold G, Poorman R, Mitchell MA. Interstitial collagenase is required for angiogenesis in vitro. Develop Biol 1994;162:499–510.

39. Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. Proc Natl Acad Sci USA 1996;93:3942–6.

40. Stenn KS, Malhotra R. Epithelialization. In: Cohen IK, Diegelmann RF, Lindblad WJ, editors. Wound healing: Biochemical and clinical aspects Philadelphia: W.B. Saunders, 1992:115–27.

41. Moore WM, Spilburg CA. Purification of human collagenases with a hydroxamic acid affinity column. Biochemistry 1986;25:5189–95.

42. Wu H, Byrne MH, Stacey A, Goldring MB, Birkhead JR, Jaenisch R, Krane SM. Generation of collagenase-resistant collagen by site-directed mutagenesis of murine pro-α1 (I) collagen gene. Proc Natl Acad Sci USA 1990 87:5888–92.

43. Guo M, Kim LT, Akiyama SK, Gralnick HR, Yamada KM, Grinnell F. Altered processing of integrin receptors during keratinocyte activation. Exp Cell Res 1991;195:315–22.

44. Cavani A, Zambruno G, Marconi A, Manca V, Marchetti M, Giannetti A. Distinctive integrin expression in the newly forming epidermis during wound repair. J Invest Dermatol 1993;101:600–4.

45. Juhasz I, Murphy GF, Yan H-C, Herlyn M, Albelda SM. Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous wound healing *in vivo*. Am J Pathol 1993;143: 1458–69.

46. Staatz WD, Rajpara SM, Wayner EA, Carter WG, Santoro SA. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{+2}-dependent adhesion of platelets to collagen. J Cell Biol 1989;108:1917–24.

47. Sudbeck BD, Parks WC, Welgus HG, Pentland AP. Collagen-mediated induction of keratinocyte collagenase is mediated by tyrosine kinase and protein kinase C activities. J Biol Chem 1994;269:30022–9.

48. Sudbeck BD, Pilcher BK, Welgus HG, Parks WC. Induction and repression of collagenase-1 by keratinocytes is controlled by distinct components of different extracellular matrix compartments. J Biol Chem 1997;272:22103–10.

49. Rudolph-Owen LA, Hulboy DL, Wilson CL, Mudgett J, Matrisian LM. Coordinate expression of matrix metalloproteinase family members in the uterus of normal, matrilysin-deficient, and stromelysin-1-deficient mice. Endocrinology 1997;138: 4902–11.

50. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell 1999;99:81–92.

51. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998;93:411–22.

52. Wilson CL, Ouellette AJ, Satchell DP, Ayabe TL, Ûpez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC. Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 1999;286:113–7.

53. Vaalamo M, Weckroth M, Puolakkainen P, Kere J, Saarinen P, Lauharanta J, Saarialho-Kere UK. Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing cutaneous wounds. Br J Dermatol 1996;135:52–9.

54. Saarialho-Kere UK, Vaalamo M, Karjalainen-Lindsberg M-L, Airola K, Parks WC, Puolakkainen P. Enhanced expression of matrilysin, collagenase, and stromelysin-1 in gastrointestinal ulcers. Am J Pathol 1996;148:519–26.

55. Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC. Matrilysin function and expression in airway epithelium. J Clin Invest 1998;102:1321–31.

56. Wang M, Qin X, Mudgett JS, Ferguson TA, Senior RM, Welgus HG. Matrix metalloproteinase deficiencies affect contact hypersensitivity: stromelysin-1 deficiency prevents the response and gelatinase B deficiency prolongs the response. Proc Natl Acad Sci USA 1999;96:6885–9.

57. Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S. Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2) -deficient mice. J Biol Chem 1997;272:22389–92.

58. Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res 1998;58:1048–51.

59. Liu Z, Shipley JM, Vu TH, Zhou X, Diaz LA, Werb Z, Senior RM. Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid. J Exp Med 1998;188:475–82.

60. Itoh T, Tanioka M, Matsuda H, Nishimoto H, Yoshioka T, Suzuki R, Uehira M. Experimental metastasis is suppressed in MMP-9-deficient mice. Clin Exp Metastasis 1999;17:177–81.

61. Wilson CL, Heppner KJ, Labosky PA, Hogan BLM, Matrisian LM. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci USA 1997;94:1402–7.

62. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema. Science 1997;277:2002–4.

Exhibit E

The EMBO Journal Vol.18 No.17 pp.4645–4656, 1999

Functional overlap between two classes of matrix-degrading proteases in wound healing

Leif R.Lund[1], John Rømer[1,2], Thomas H.Bugge[3,4], Boye S.Nielsen[1], Thomas L.Frandsen[1], Jay L.Degen[3], Ross W.Stephens[1] and Keld Danø[1,5]

[1]The Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark and [3]Division of Developmental Biology, Children's Hospital Research Foundation, Cincinnati, OH 45229, USA

[2]Present address: Histology, Health Care Discovery, Novo Nordisk, 2880 Bagsværd, Denmark
[4]Present address: National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA

[5]Corresponding author
e-mail: keld.dano@finsenlab.dk

L.R.Lund and J.Rømer contributed equally to this work

Retarded wound healing was found in mice deficient in the serine protease precursor plasminogen, as well as in wild-type mice treated with the metalloprotease inhibitor galardin, but in both cases wound closure was ultimately completed in all mice within 60 days. The expression of several matrix metalloproteases in keratinocytes migrating to cover the wound was strongly enhanced by galardin treatment. However, when plasminogen-deficient mice were treated with galardin, healing was completely arrested and wound closure was not seen during an observation period of 100 days, demonstrating that protease activity is essential for skin wound healing. The requirement for both plasminogen deficiency and metalloprotease inhibition for complete inhibition of the healing process indicates that there is a functional overlap between the two classes of matrix-degrading proteases, probably in the dissection of the fibrin-rich provisional matrix by migrating keratinocytes. Each class alone is capable of maintaining sufficient keratinocyte migration to regenerate the epidermal surface, although this function would normally be performed by both classes acting in parallel. Since there are strong similarities between the proteolytic mechanisms in wound healing and cancer invasion, these results predict that complete arrest of this latter process in therapeutic settings will require the use of inhibitors of both classes of proteases.
Keywords: keratinocytes/metalloprotease/plasminogen/serine protease/wound healing

Introduction

Wound healing is a tissue remodeling process in which the injured tissue is removed and substituted with normal tissue. Proteolytic degradation of the extracellular matrix is considered to play a crucial role in this process (Grøndahl-Hansen *et al.*, 1988; Werb, 1997) as well as in a variety of other physiological and pathological processes involving tissue remodeling and cell migration. These include trophoblast invasion (Sappino *et al.*, 1989; Rinkenberger *et al.*, 1997; Teesalu *et al.*, 1998), post-lactational mammary gland involution (Talhouk *et al.*, 1992; Lund *et al.*, 1996), angiogenesis (Pepper *et al.*, 1987; Mignatti *et al.*, 1996), inflammation (Werb, 1997) and cancer invasion (Danø *et al.*, 1985, 1994; Liotta *et al.*, 1991; Blasi, 1993). Several proteolytic enzymes such as the serine protease plasmin and a number of matrix metalloproteases (MMPs) are thought to be involved in the degradation of extracellular matrix (Sternlicht and Werb, 1999). This assumption is mostly based on correlative observations and *in vitro* studies. Recently, however, a role for plasminogen (Plg), the precursor of plasmin, in a tissue remodeling process was demonstrated conclusively by the finding of impaired skin wound healing in mice with a disrupted Plg gene (Rømer *et al.*, 1996). In these mice, there was a decreased rate of migration of keratinocytes from the wound edge during the re-epithelialization process, which we proposed was due to a diminished ability of these cells to dissect their way proteolytically through the fibrin-rich extracellular matrix beneath the wound crust. Strong support for this interpretation comes from the observation that virtually normal skin wound healing time is restored in mice deficient in both Plg and fibrin (Bugge *et al.*, 1996).

Although wound healing in Plg-deficient mice is impaired, wound closure eventually is achieved. In our previous studies, complete healing of all wounds occurred within 13 days in wild-type mice, while 60% of the wounds in the Plg-deficient mice were healed at the termination of the experiment at day 50 (Rømer *et al.*, 1996). In subsequent experiments (see below), all wounds in Plg-deficient mice were healed by around day 60. During healing of skin wounds, the migrating keratinocytes express key mediators and regulators of plasminogen activation, including urokinase Plg activator (uPA), uPA receptor (uPAR) and type 1 PA inhibitor (PAI-1) (Rømer *et al.*, 1991, 1994). In addition, they express several MMPs (Hewitt and Danø, 1996; see also below). We therefore hypothesized that the MMPs may also play a role in matrix degradation during wound healing, that there is a functional overlap between plasmin and the MMPs, and that the residual wound healing capacity observed in the Plg-deficient mice reflects the ability of MMPs to perform alone the functions which they would perform together with plasmin in wild-type mice. In order to test this hypothesis, we have now studied the effect of galardin, a hydroxamate inhibitor of a broad spectrum of MMPs (Grobelny *et al.*, 1992; Levy *et al.*, 1998), on skin wound healing in wild-type and Plg-deficient mice.

Results

Several MMPs are expressed during skin wound healing

To examine the expression of MMPs during skin repair, full thickness incisional wounds were made in wild-type mice (n = 12), and sections were analyzed microscopically at time points ranging from 12 h to 7 days after wounding. mRNAs encoding the respective proteases were detected by *in situ* hybridization with probes specific for seven different MMPs, including gelatinase A (MMP-2), gelatinase B (MMP-9), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), MT-1 MMP (MMP-14) and macrophage metalloelastase (MMP-12). In normal skin, there was no detectable mRNA for any of these MMPs, while they were all expressed in the skin wounds.

Gelatinase B mRNA was detected already 12 h after the wounding in the keratinocytes at the wound edge, which had just begun to flatten at that time and to move into and under the wound crust. All these gelatinase B-expressing cells were located basally just at the front of the moving epidermal layer. At all subsequent time points until 7 days after wounding, gelatinase B mRNA was found in keratinocytes at the same location (Figure 1a and b). Collagenase-3 mRNA was expressed in a pattern similar to that of gelatinase B, the only difference being that collagenase-3 mRNA was also detected in many basal keratinocytes located further behind the leading edge (Figure 1e and f). Expression of gelatinase B mRNA was not detected in the granulation tissue or in any cell types other than the keratinocytes, while collagenase-3 mRNA was found occasionally in macrophage-like cells in the granulation tissue. When the wounds were completely covered with a newly formed epidermal layer, expression of mRNA for the two MMPs was no longer detectable in any cells in the skin. mRNA for gelatinase A, stromelysin-3 and MT-1 MMP were all detected in fibroblasts located in the lower dermis at the edge of the wound site from 12 h after wounding. At later time points, strong expression of mRNA from these three genes was also seen in the granulation tissue, located in fibroblast-like cells which were migrating under the wound site. Comparison of adjacent sections showed that gelatinase A and MT-1 MMP mRNA were expressed in the very same fibroblasts (not shown). In the last phase of the healing processes leading to closure of the wound site with epidermis, expression of these three MMPs was seen in fibroblast-like cells located very close to the newly formed epidermal basement membrane. After the closure of the wound site with a new epidermal layer, weak signals for gelatinase A, stromelysin-3 and MT-1 MMP mRNA were still found in some fibroblast-like cells. No expression of mRNAs for any of these three molecules was detected in keratinocytes or in any cells other than fibroblast-like cells in the wounds.

Stromelysin-1 and macrophage metalloelastase each had a unique expression pattern. Stromelysin-1 mRNA was found in both keratinocytes and fibroblasts. The expression was strongest in the basal keratinocytes behind the migrating front in the area corresponding to the initial wound edge, and in fibroblasts located below and adjacent to these keratinocytes, but stromelysin-1 mRNA was also detected in the leading edge keratinocytes (Figure 1c and d). Macrophage metalloelastase mRNA was expressed in a subset of macrophages located below and adjacent to the wound crust (not shown). Expression of stromelysin-1 and macrophage metalloelastase mRNA was not detected in any other cells in the wounds.

Delay of wound healing by treatment with an MMP inhibitor

The expression of several MMPs in leading edge keratinocytes during wound healing is remarkably similar to the expression we have found previously for the key components of the plasminogen activation system, uPA and uPAR (Rømer *et al.*, 1991, 1994, 1996), which could suggest that some of the MMPs, like plasmin, are involved in matrix proteolysis in the healing process. To test this hypothesis, we made standardized 20 mm long, full-thickness incisional wounds in the skin of mice, and then treated the mice with daily intraperitoneal injections of increasing doses of the metalloprotease inhibitor galardin. The wounds were examined visually and the wound length measured the day after surgery and at 2–3 day intervals thereafter. Wounds were scored as fully healed when there was a loss of the wound crust and a macroscopic closure of the incision interface with restoration of the epidermal covering. All wounds were spindle-shaped immediately after incision, with well-separated incision edges and exposure of the underlying muscle fascia. By the second day, all wounds were covered with a dry wound scab. In control mice mock treated with carboxymethylcellulose (CMC), the vehicle used for suspension of galardin, there was a gradual loss of the scab and decrease in the wound length, until all wounds were healed at day 15 (Figure 2). In the galardin-treated mice, there was a delay in the healing, which became more pronounced with higher doses of the inhibitor and was statistically highly significant with doses of 50 mg/kg and above (P <0.0001 in all cases). Thus at the termination of the experiment at day 25, only 30% of the wounds were healed in mice treated daily with 100 mg/kg of galardin. The remaining wounds were covered with a dry scab and had thickened edges. As described in further detail below (see the section Keratinocyte migration and Figure 4), a microscopic analysis of the wounds revealed that the rate of migration of keratinocytes to cover the wound field was significantly inhibited by galardin treatment, probably causing the observed delay in wound closure.

Wound healing is arrested completely in Plg-deficient mice treated with MMP inhibitor

The effect of galardin on wound healing in wild-type mice is thus very similar to that of Plg deficiency caused by targeted gene inactivation, consistent with the hypothesis of similar and overlapping functions of plasmin and one or more MMPs in this process. A prediction from this hypothesis would be that an additive and perhaps synergistic effect could be produced if Plg deficiency is combined with MMP inhibition.

This prediction pre-supposes that the Plg deficiency does not in itself lead to a substantial up-regulation of MMP expression. In order to evaluate this possibility, we examined the expression of mRNAs for the seven MMPs discussed above by *in situ* hybridization of sections from

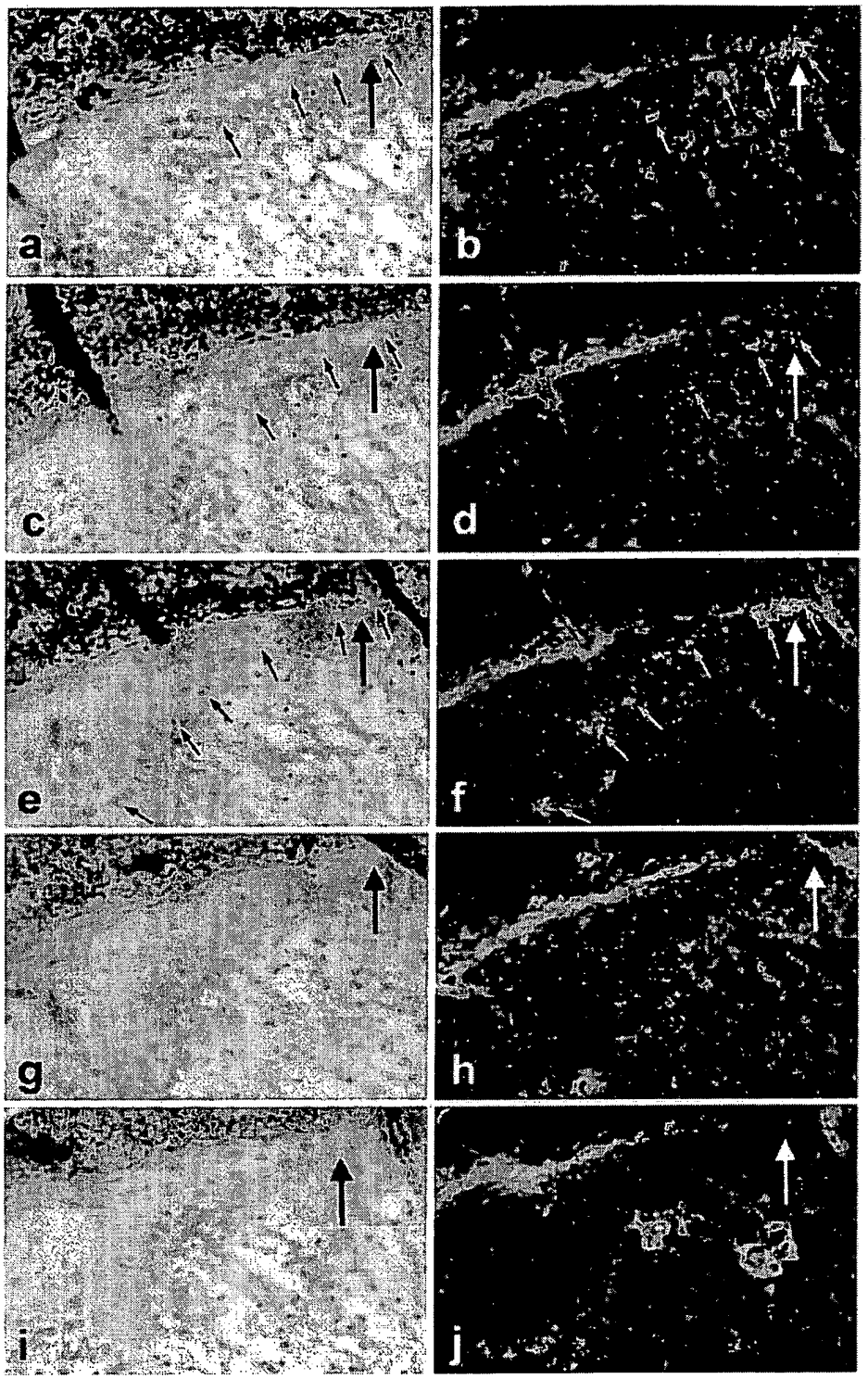


Fig. 1. Metalloprotease expression during skin wound healing. Sections of wounded skin removed 7 days after surgery of wild-type mice were analyzed by *in situ* hybridization for expression of mRNA for gelatinase B (**a** and **b**), stromelysin-1 (**c** and **d**), collagenase-3 (**e** and **f**), gelatinase A (**g** and **h**) and stromelysin-3 (**i** and **j**). The large straight arrows indicate the tip of the epidermal tongue. The small straight arrows indicate expression of gelatinase B, stromelysin-1 and collagenase-3 mRNA in the migrating keratinocytes. Note that mRNAs for gelatinase B, stromelysin-1 and collagenase-3 are expressed in the leading edge keratinocytes, whereas gelatinase A and stromelysin-3 mRNAs could not be detected in the keratinocytes. (a), (c), (e), (g) and (i) are bright-field and (b), (d), (f), (h) and (j) are dark-field micrographs. Magnification, ×160.

skin wounds derived from our previous study of wound healing in mice homozygous for a disrupted Plg gene ($Plg^{-/-}$ mice) (Rømer *et al.*, 1996). Littermates that were homozygous for the wild-type Plg gene ($Plg^{+/+}$ mice) were included as controls. All seven mRNAs were expressed in the $Plg^{-/-}$ mice in a pattern and to a degree similar to

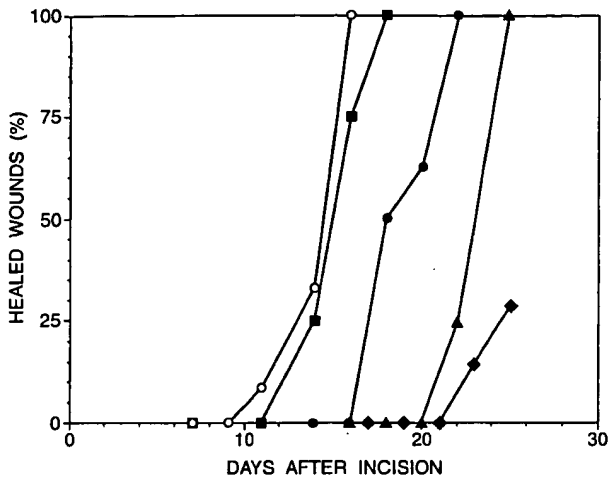


Fig. 2. Effect of the MMP inhibitor galardin on skin wound healing in wild-type mice. The percentage fractions of mice with healing defined as loss of the wound scab and complete covering of the epidermis are plotted versus time after the incision, for mice treated i.p. with galardin in daily doses of 25 mg/kg (■), 50 mg/kg (●), 75 mg/kg (▲) and 100 mg/kg (◆) or mock-treated with 4% CMC in 0.9% saline (○), the vehicle used for suspension of galardin.

that described above for wild-type mice. This pattern of expression was also observed in the $Plg^{+/+}$ littermate control mice, taking into account that the wound healing is strongly delayed in the Plg-deficient mice, i.e. at the same stage of healing the MMP mRNA expression observed in mice of the two genotypes was similar with respect to both location and intensity of the respective hybridization signals. We thus observed no sign of transcriptional up-regulation of MMP genes in the Plg-deficient mice.

We then treated wounded $Plg^{-/-}$ mice with galardin (Figure 3). As controls, we included littermate $Plg^{+/+}$ mice and groups of mice of each genotype that were given mock treatment with the vehicle CMC alone. In this experiment, we used mice backcrossed into NIHS mice. Wild-type mice of this genetic background had a somewhat slower wound healing than the C57BL/6J mice used in the experiment shown in Figure 2, and the effect of galardin on this process was less pronounced; 100 mg/kg only causing a moderate and statistically insignificant ($P = 0.06$) delay. In the mock-treated Plg-deficient mice, there was a stronger and statistically highly significant ($P < 0.0001$) delay in healing, similar to what we found in our previous study. The macroscopic appearance of the wounds was similar in galardin-treated wild-type mice and mock-treated Plg-deficient mice. These wounds were characterized by a long period with a gaping and red wound field, which had a hard and scaly surface, lacked epidermal covering, and often granulation tissue protruded between the wound edges. However, in both groups, all wounds were healed at approximately day 60 (Figure 3b). The healed skin in these two groups differed from that in the mock-treated wild-type mice in that it was palpably thicker than the surrounding skin, indicating an abnormal healing process.

In the group of mice which were both Plg-deficient and treated with galardin, all wounds remained open within

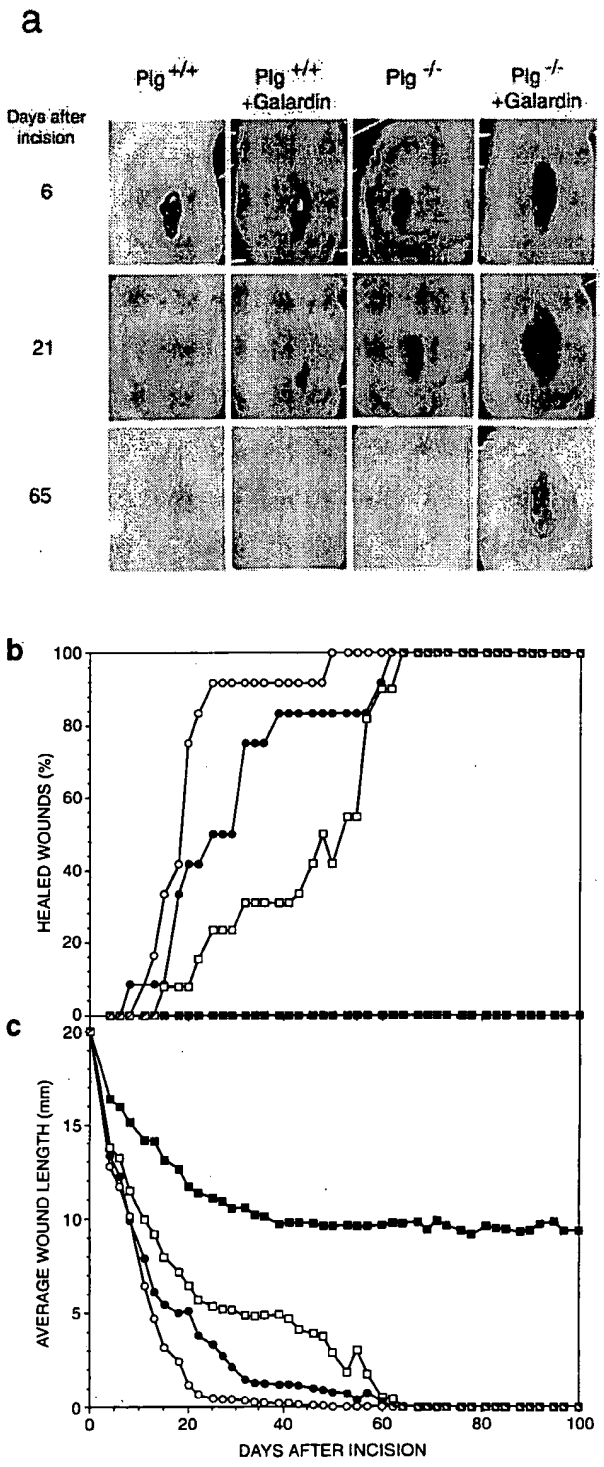


Fig. 3. Combined effect of Plg deficiency and MMP inhibition on skin wound healing. (a) Examples of the progress of repair processes in wild-type and Plg-deficient mice with and without galardin treatment. (b) The percentage fractions of mice with healing are plotted versus time after the incision for wild-type mice that were mock-treated with CMC (○) or treated daily i.p. with 100 mg/kg of galardin (●) and Plg-deficient mice that were mock-treated (□) or treated daily i.p. with 100 mg/kg of galardin (■). (c) The average lengths of the healing incisional wounds are plotted versus time for the same groups of mice. The mice in this experiment were obtained by backcrossing of Plg gene-targeted mice to NIHS mice for six generations.

the observation period of 100 days (Figure 3b), in stark contrast to the complete wound healing eventually observed in all of the mice which were either deprived of Plg or treated with the MMP inhibitor. Combination of Plg deficiency and MMP inhibition thus had a synergistic effect on the overall wound healing process. The lack of complete healing in the galardin-treated Plg-deficient mice was also reflected in the length of the wounds, which initially decreased, but then from approximately day 30 after incision remained stable at around half of the initial length (Figure 3c), the wounds being broad with a hard, dry and scaly surface. We did not observe any effect of Plg deficiency, galardin treatment or both on other parameters that potentially could influence the healing process, such as initial dryness of the wound bed, skin contraction or infection.

In a separate experiment with C57Bl/6J mice and a 42 day treatment and observation period, similar results were obtained. At day 42, no wounds were healed in Plg-deficient C57Bl/6J mice treated daily with 100 mg/kg of galardin, 60% were healed in mock-treated Plg-deficient mice and 56% in galardin-treated, littermate, wild-type mice, while all wounds in mock-treated, littermate, wild-type mice were healed at day 18.

Keratinocyte migration

In a separate experiment, we analyzed the impact of galardin treatment on keratinocyte migration in wild-type and littermate Plg-deficient mice. Groups of the two genotypes were either treated with 100 mg/kg daily of galardin for 7 days after skin wounding or mock treated. Keratin-stained sections of wound tissue from each group of mice were then analyzed microscopically, and the length of the epidermal tongue along its base from the wound edge to the tip of the wedge (see Figure 4) was measured blindly in three or four sections per wound by computer-assisted morphometry. This distance included both the actual migration distance covered by the keratinocytes and the epidermal area just behind, consisting of proliferating keratinocytes. In the mock-treated wild-type mice (n = 5), the length of the epidermal tongue was 1227 ± 114 μm (mean value ± SEM), compared with 908 ± 119 μm in the galardin-treated wild-type mice (n = 5), 950 ± 217 μm in the mock-treated Plg-deficient mice (n = 4) and 514 ± 74 μm in the galardin-treated Plg-deficient mice mice (n = 5). Evaluated by a nested analysis of variance (ANOVA), the decrease in the length of the epidermal tongue caused by galardin treatment of wild-type mice was statistically significant and this was also the case for the decrease caused by Plg deficiency alone (P <0.01 in both cases). In the galardin-treated Plg-deficient mice, the decrease was significant in comparison with the mock-treated wild-type mice, the galardin-treated wild-type mice and the mock-treated Plg-deficient mice (P <0.01 in all cases). Thus, MMP inhibition and Plg deprivation each reduced the length of the epidermal tongue, and in combination had at least an additive effect on this parameter.

In the galardin-treated Plg-deficient mice, the tip of the epidermal tongue was located very close to the initial wound edge, identified as the area just beneath the edge of the wound crust. The measured length of the epidermal tongue in these mice therefore represented almost entirely the zone of proliferation behind the leading edge keratinocytes, reflecting that the keratinocyte migration was virtually blocked. Staining of the wound sections with Masson-trichrome to reveal the collagen distribution showed that in both the mock-treated and galardin-treated wild-type mice, the epidermal outgrowth was wedge shaped and surrounded by a loose network of the provisional extracellular matrix (Figure 4a–d). Occasionally, the epidermal tongue in the galardin-treated wild-type mice was surrounded by a dense collagenous matrix (not shown). In the mock- and galardin-treated Plg-deficient mice, the epidermal tongues were blunted and surrounded by a dense layer of fibrillar non-collagenous material (curved arrows in Figure 4e–h). This layer was most pronounced in the galardin-treated Plg-deficient mice.

Immunohistochemical staining for fibrin(ogen) revealed that increased amounts of fibrin were present below and in front of the epidermal outgrowths in all of the galardin-treated wild-type mice (Figure 5c), the mock-treated Plg-deficient mice (Figure 5b) and the galardin-treated Plg-deficient mice (Figure 5d), compared with the mock-treated wild-type (Figure 5a) mice (n = 5 in each group). It is particularly noteworthy that the compact and band-shaped fibrin immunoreactivity was found below and in front of the keratinocytes in all five wild-type mice treated with galardin, whereas such a pattern of fibrin immunoreactivity was absent in four out of five mock-treated wild-type mice (Figure 5a shows a representative example). These findings indicate that galardin treatment alone, like Plg deficiency alone, may impair keratinocyte migration by hampering the degradation of fibrin immediately in front of the leading edge keratinocytes. We also immunohistochemically investigated the localization of fibronectin and laminin in the wounds, but found no obvious differences in the immunostaining patterns of these proteins between mock- and galardin-treated wild-type and Plg-deficient mice.

In the galardin-treated wild-type mice, and both the mock- and galardin-treated Plg-deficient mice, there was abundant formation of granulation tissue with inflammatory cells, endothelial cells and fibroblasts (Figure 4). We did not observe any apparent signs of reduced angiogenesis in the wounds from any of these three groups of mice.

Increased MMP expression after treatment with MMP inhibitor

Seven days after surgery, the expression of gelatinase B, collagenase-3, stromelysin-1, stromelysin-3 and gelatinase A was examined by *in situ* hybridization of wound tissue from mock-treated Plg-deficient and wild-type mice, and from mice of both genotypes that had been treated with daily doses of 100 mg/kg of galardin. In the mock-treated mice, the expression of these MMPs was as described above for untreated animals (compare Figure 6a and e). However, in both wild-type and Plg-deficient mice treated with galardin, the expression of gelatinase B (Figure 6a, e, f and j), stromelysin-1 (Figure 6b and g) and collagenase-3 (Figure 6c and h) mRNA was dramatically up-regulated in the leading edge keratinocytes compared with the mock-treated mice of the same genotype. Expression of gelatinase B, stromelysin-1, collagenase-3 and gelatinase A was also up-regulated in both groups of galardin-treated mice in the granulation

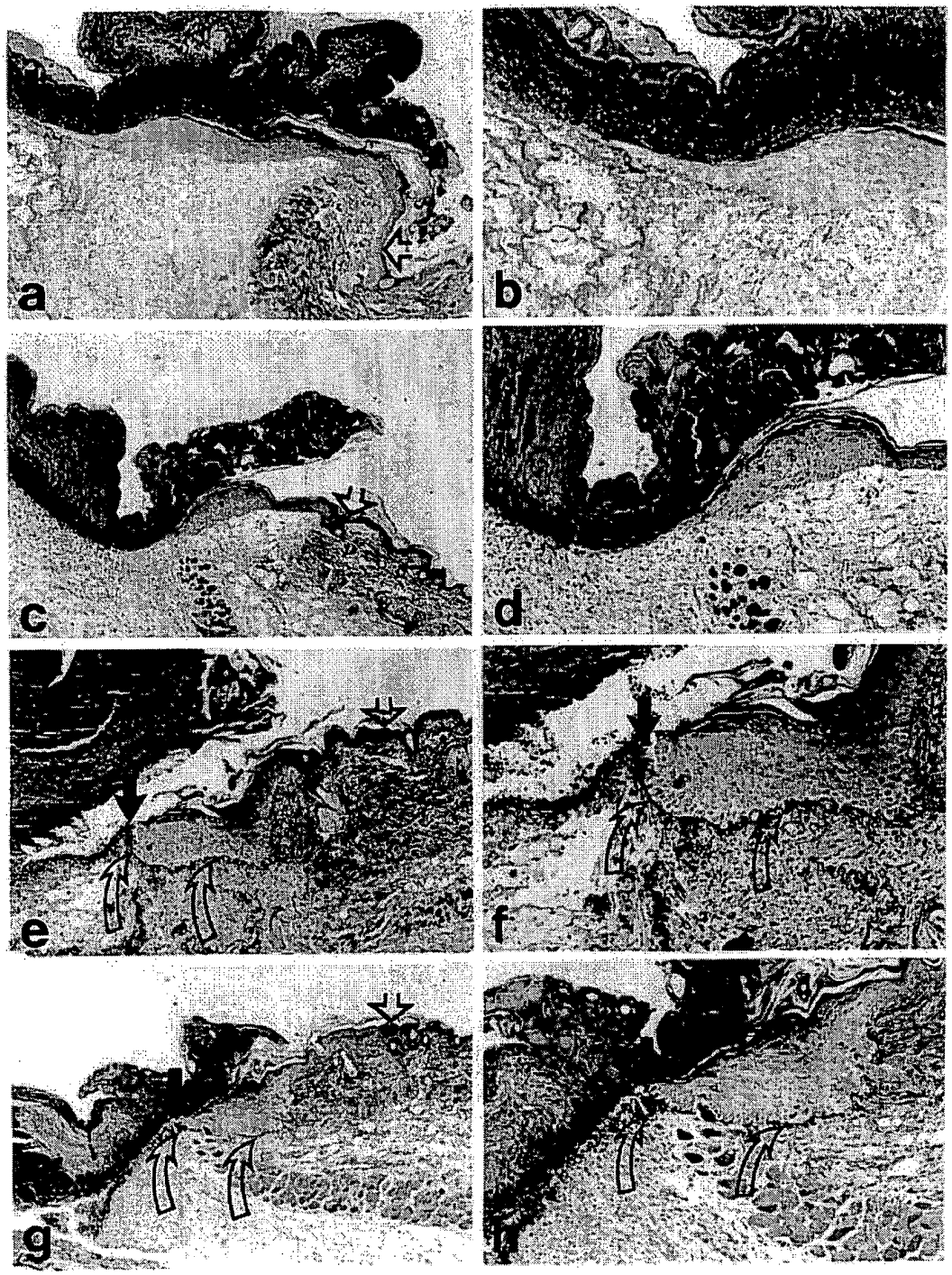


Fig. 4. Microscopic appearance of skin wounds in mock- and galardin-treated Plg-deficient and wild-type mice. Sections of skin wounds 7 days after surgery were stained with Masson-trichrome. In mock-treated (**a** and **b**) and galardin-treated (**c** and **d**) wild-type mice, the epidermal tongue is wedge shaped and surrounded by a loose network of extracellular matrix. In mock-treated (**e** and **f**) and galardin-treated (**g** and **h**) Plg-deficient mice, the epidermal ends are blunted and surrounded by a dense layer of fibrillar material. The fibrillar material is shown by curved arrows in (e–h) and is most pronounced in the galardin-treated Plg-deficient mice (h). In (a), (c), (e) and (g), the straight closed arrows indicate the tip of the epidermal tongue and the straight open arrows indicate the point at the start of the zone of proliferating keratinocytes used for measurements of the length of the epidermal tongue. (b), (d), (f) and (h) are larger magnifications of (a), (c), (e) and (g), respectively. Magnification: (a), (c), (e) and (g), ×45; (b), (d), (f) and (h), ×90.

tissue located just beneath the leading edge keratinocytes (curved arrows in Figure 6b–d and f–i). Stromelysin-1 and gelatinase A were expressed in fibroblast-like cells in the galardin-treated mice, as in the mock-treated mice, while gelatinase B and collagenase-3 were expressed in macrophage-like cells in the galardin-treated mice. Furthermore, expression of both gelatinase A and stromelysin-3 mRNA was up-regulated in fibroblast-like

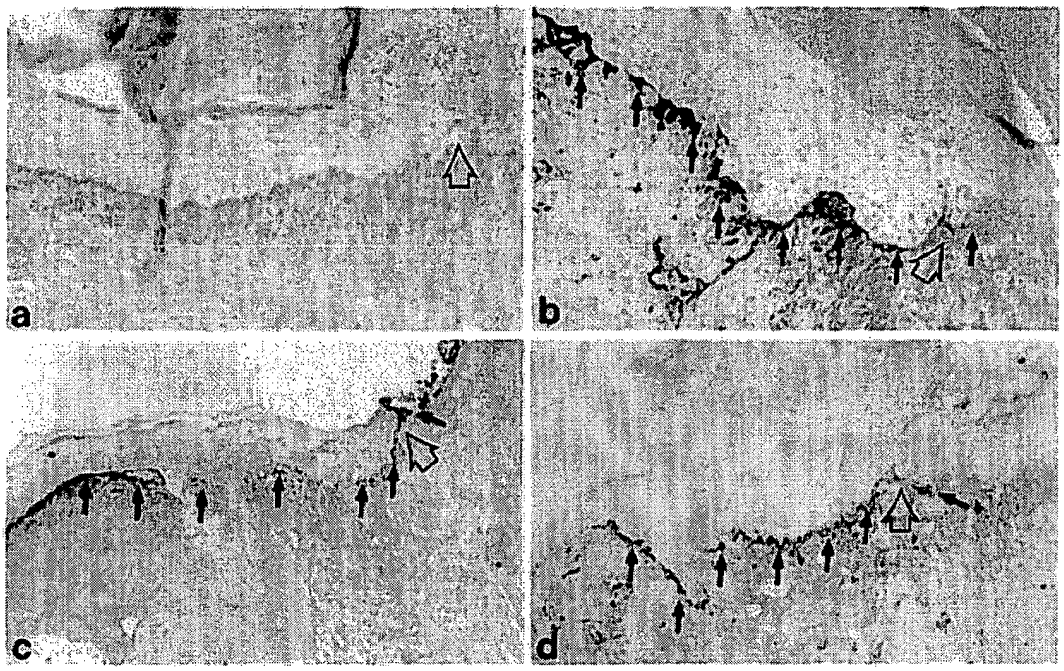


Fig. 5. Immunohistochemical staining for fibrin in wounds from $Plg^{+/+}$ and $Plg^{-/-}$ mice treated with galardin or vehicle 7 days after surgery. Sections from mock-treated wild-type (**a**) and plasminogen-deficient (**b**) mice, and from galardin-treated wild-type (**c**) and plasminogen-deficient (**d**) mice were stained immunohistochemically for fibrin(ogen). Both in the wild-type mice treated with galardin and in the Plg-deficient mice treated with either vehicle or galardin, fibrillar deposits are seen containing fibrin in front of and below the epidermal outgrowth (small straight arrows). There is no apparent difference in the intensity of the fibrin staining between the wild-type mice treated with galardin and the Plg-deficient mice treated with either vehicle or galardin. The open arrows in (a–d) show the ends of the epidermal outgrowth. Magnification, ×170.

cells in the deep part of the granulation tissue bordering the normal subcutis in the galardin-treated wild-type and Plg-deficient mice (not shown). Gelatinase A mRNA expression in these animals was also detectable in the leading edge keratinocytes, in contrast to the lack of detectable gelatinase A expression in keratinocytes in the mock-treated mice (Figure 6d and i).

Discussion

The findings in the present study demonstrate that treatment of mice with a metalloprotease inhibitor, galardin, impairs skin wound healing in mice. We have reported previously that Plg deficiency achieved by targeted gene disruption results in a similar delay in wound repair. In both cases, the repair process does, however, eventually proceed, leading to complete healing in all mice. In contrast, the wound healing process is effectively brought to a standstill and complete wound healing is not observed in any Plg-deficient mice treated with galardin, thus demonstrating that protease activity is essential for this tissue remodeling process, and that there is a synergistic effect of Plg deficiency and galardin treatment.

In the case of both Plg deficiency and galardin treatment, there is an impairment of the migration of keratinocytes from the wound edge, while we did not observe any decrease in the formation of granulation tissue or in neovascularization in any of the cases. With respect to Plg deficiency, a reversal to virtually normal skin wound healing time in mice made deficient in both Plg and fibrinogen (Bugge *et al.*, 1996) indicates that the main reason for the impairment is a decreased ability of the Plg-deprived keratinocytes to dissect their way proteolytically through the fibrin-rich extracellular matrix beneath the wound crust. Our present finding of excessive amounts of fibrin below and in front of the migrating keratinocytes in galardin-treated wild-type mice makes it likely that the mechanism of action of the galardin-induced impairment of wound healing is a similar inhibition of degradation of fibrin and possibly other matrix components by the migrating keratinocytes. In favor of this proposal is the finding that these keratinocytes express not only the key regulators of Plg activation, uPA and uPAR, but also the three MMPs gelatinase B, collagenase-3 and stromelysin-1. Moreover, at least two MMPs, stromelysin-1 (Bini *et al.*, 1996) and MT-1 MMP (Hiraoka *et al.*, 1998), have been shown to degrade fibrin, and in the latter case the data strongly argue that in some tissue remodeling processes MMPs may even be more important than plasmin in providing the fibrinolytic activity required for cell migration (Hiraoka *et al.*, 1998).

Galardin is a peptide-based zinc-chelating hydroxamate that inhibits the proteolytic activity of several MMPs, including gelatinase A (0.5 nM), gelatinase B (0.2 nM), interstitial collagenase (0.4 nM), neutrophil collagenase (0.1 nM) and stromelysin 1 (30 nM) with the K_is indicated (Grobelny *et al.*, 1992; Levy *et al.*, 1998). In at least four studies of systemic galardin treatment in animal models, specific effects were observed which, as in the present study, most probably were due to inhibition of MMP-mediated degradation of the extracellular matrix, while there were no observations of general toxicity (Galardy *et al.*, 1994; Gijbels, 1994; Strauss *et al.*, 1996; Witte *et al.*, 1998). However, it cannot be excluded at present that the inhibition of metalloprotease-mediated activation of latent growth factors by galardin may also

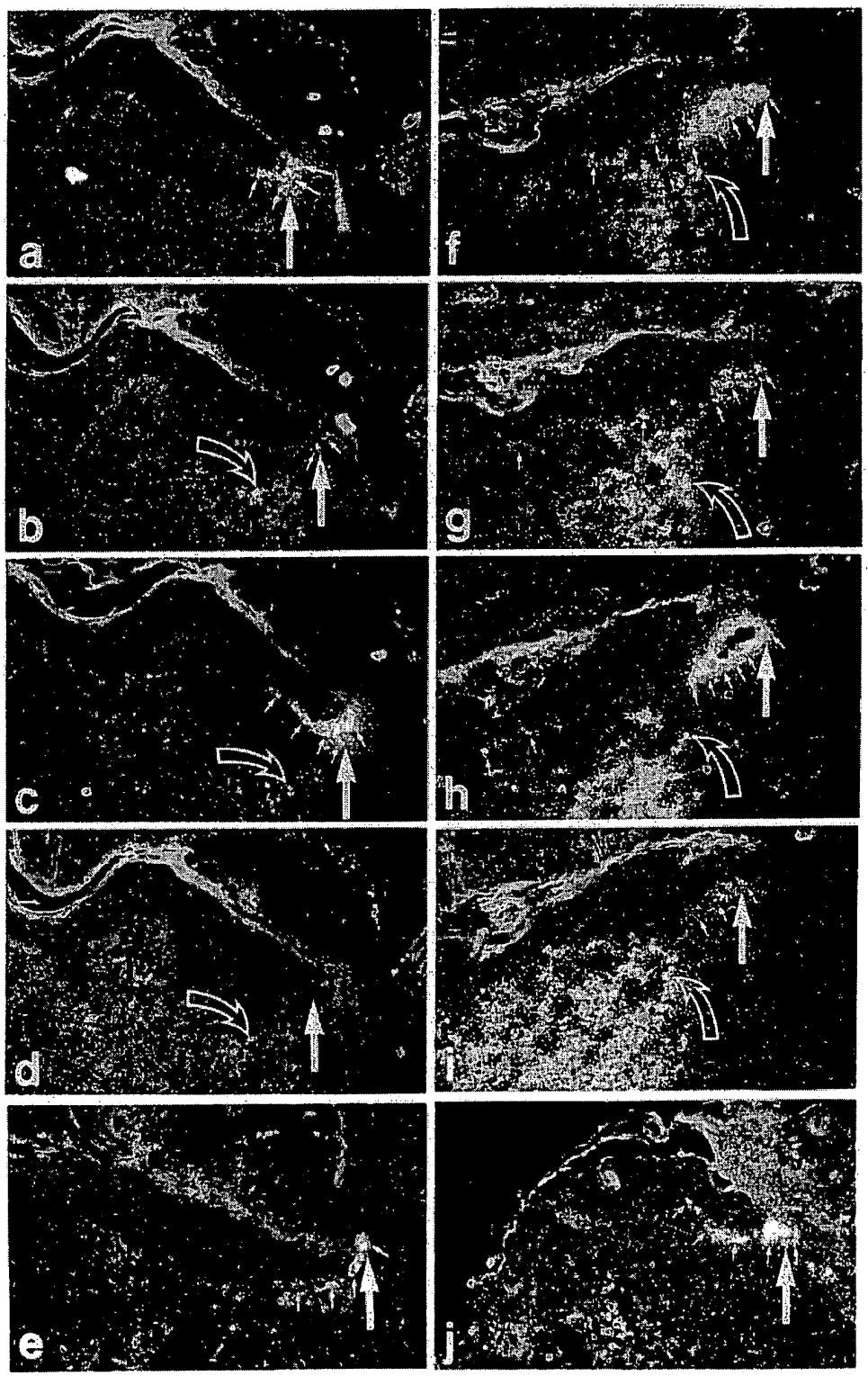


Fig. 6. Increased metalloprotease expression after treatment with galardin. The expression of metalloproteases in skin wounds from mock-treated (a–e) or galardin-treated (f–j) Plg-deficient (a–d and f–i) or wild-type (e and j) mice were analyzed by *in situ* hybridization 7 days after surgery. The expression of mRNA for gelatinase B (**a**, **e**, **f** and **j**), stromelysin-1 (**b** and **g**), collagenase-3 (**c** and **h**) and gelatinase A (**d** and **i**) is increased in all the galardin-treated animals. The large straight arrows indicate the tip of the epidermal tongue. The small straight arrows show expression of gelatinase B (a, e, f and j), stromelysin-1 (b and g) and collagenase-3 (c and h) in the leading edge keratinocytes of both the mock- and galardin-treated Plg-deficient or wild-type mice, and of gelatinase A (i) in the tip of the leading edge keratinocytes in the galardin-treated animals. The curved arrows indicate expression at the front of the granulation tissue of gelatinase B (f) in galardin-treated Plg-deficient mice, and of stromelysin-1 (b and g), collagenase-3 (c and h) and gelatinase A (d and i) in both mock- and galardin-treated Plg-deficient animals. Note that the expression of all four MMPs is up-regulated both in the leading edge keratinocytes and in the granulation tissue cells in the galardin-treated animals. All micrographs are dark-field images. Magnification, ×90.

contribute to the observed impairment of keratinocyte migration. Indeed, a recent report provides evidence that some functional inhibition of tumor necrosis factor-α (TNF-α)-converting enzyme (TACE)—a metalloprotease disintegrin (Wolfberg *et al.*, 1995; Moss *et al.*, 1997)—may be achieved *in vivo* by systemic galardin treatment of mice (Solorzano *et al.*, 1997). It should be noted, however, that deficiency in one of the principle substrates of TACE, transforming growth factor-α (Peschon *et al.*, 1998), did not affect keratinocyte migration or other components of the healing process in a skin wound healing model similar to the one applied in this study (Guo *et al.*, 1996). Furthermore, to our knowledge, effects on skin wound healing of deficiencies in other substrates of TACE, such as the TNF-α–TNF-α receptor complex and L-selectin, have not been reported.

It is noteworthy that we found a strong increase in the expression of several MMPs in the keratinocytes after treatment with galardin, indicating that the galardin was bioavailable at the wound site, and could exert an effect there. However, this up-regulation also suggests that there are compensatory regulatory systems, whereby the persistence of specific matrix components increases MMP gene expression. The mechanism of the up-regulation is yet to be clarified, but recent reports on collagen V-induced up-regulation of MMP-1 expression through an interaction with the discoidin domain receptor 2 indicate that such feedback mechanisms exist (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997). However, due to the number of MMPs that are known to be inhibited by galardin, we are unable to specify which MMPs are critically involved in wound healing. In preliminary experiments with mice deficient in either gelatinase B or stromelysin-1, we have not found any retardation of wound healing (unpublished results), and it appears likely that several of the at least 17 known MMPs need to be inactivated concomitantly in order to obtain an impairment of the healing process.

The observation that wound healing does proceed in the absence of Plg indicates that alternative mechanisms exist which can perform the function of plasmin. The complete arrest obtained with galardin treatment in Plg-deficient mice, but not in wild-type mice, shows that these alternative mechanisms involve metalloprotease activity. We therefore conclude that there are one or more functions that can be exerted by either of the two classes of proteases. The lack of detectable up-regulation of metalloprotease expression in the Plg-deficient mice makes it likely that the metalloproteases also exert this function in wild-type mice, and that there is normally a functional overlap between the two classes of proteases. A probable explanation for the functional overlap in skin wound healing demonstrated in this study is that cleavage of one or more extracellular matrix components is critical for keratinocyte migration, and that this cleavage can be achieved by either of the two protease systems. Our previous studies on Plg-deficient mice (Rømer *et al.*, 1996), together with studies of mice with combined deficits in Plg and fibrinogen (Bugge *et al.*, 1996), indicate that degradation of fibrin is a critical function of plasmin in skin wound repair. As noted above, our present findings of residual fibrin-rich matrix in galardin-treated mice argue that MMPs also contribute to fibrin degradation by migrating keratinocytes, so that fibrin may be a target for both plasmin and MMPs. In view of these findings, the slow but ultimately successful repair of wounds in Plg-deficient mice is likely to be due to slow clearance of fibrin by MMPs. We did not find any detectable changes in the staining of fibronectin and laminin, which are also known to be substrates for both plasmin and MMPs *in vitro* and, probably, *in vivo* (Danø *et al.*, 1985; Murphy *et al.*, 1992; Chen and Strickland, 1998; Tsirka *et al.*, 1998). We cannot, however, exclude that insufficient cleavage of these or other matrix proteins, such as vitronectin and proteoglycans (Whitelock *et al.*, 1996; Waltz *et al.*, 1997), may also contribute to the complete arrest of wound healing that we observed in galardin-treated Plg-deficient mice. The hypothesis that one or more matrix components are common substrates for plasmin and MMPs, and that this explains their functional overlap in wound healing, predicts that the healing would be restored in Plg-deficient, galardin-treated mice if the animals also lacked one or more of those matrix components. With respect to fibrin, this prediction can be tested using available fibrinogen knockout mice (Bugge *et al.*, 1996).

In vitro observations suggest that plasmin plays an important role in the activation of some pro-metalloproteases (Murphy *et al.*, 1992; Mazzieri *et al.*, 1997). The pronounced effect of galardin treatment on wound healing in Plg-deficient mice observed in this study does, however, clearly demonstrate that at least some metalloproteases involved in wound healing are not dependent on plasmin activation in order to promote tissue remodeling. This conclusion is supported further by the finding that active gelatinase B, as identified by zymography (see Lund *et al.*, 1996), is present in extracts of wound tissue from Plg-deficient mice (unpublished results). The arrest of wound healing seen in galardin-treated Plg-deficient mice, but not in galardin-treated wild-type mice, argues that Plg has more functions in tissue remodeling than activation of MMPs.

The present findings may have implications not only for our understanding of the proteolytic mechanisms involved in wound healing, but also for other normal and pathological tissue remodeling processes, including trophoblast invasion, organ involution, inflammatory diseases and in particular cancer invasion. It is becoming increasingly clear that the same repertoire of proteases is involved in degradation of the extracellular matrix in such processes (Danø *et al.*, 1985; Hewitt and Danø, 1996; Edwards *et al.*, 1998; Johnsen *et al.*, 1998), and that there are also strong similarities in the way in which epithelial cells interact with stromal connective tissue cells in the generation and regulation of extracellular proteolysis (Hewitt and Danø, 1996; Johnsen *et al.*, 1998). Particularly striking are the similarities between wound healing and cancer (Dvorak *et al.*, 1986). These similarities were extended recently to findings in Plg-deficient mice in which, besides impaired wound healing (Rømer *et al.*, 1996), there is also an impairment of primary growth of the transplanted Lewis lung carcinoma (Bugge *et al.*, 1997) and of metastasis of mammary tumors genetically induced by overexpression of the polyoma virus middle T antigen (Bugge *et al.*, 1998). Like the skin wound healing, tumor growth and metastasis do, however, eventually proceed in the absence of Plg, probably reflecting the notion that also in these processes there is a functional

overlap between plasmin and metalloproteases. The present findings predict that effective arrest of cancer progression will require the combined use of inhibitors of MMPs and inhibitors of the plasmin/Plg activation system. Several MMP inhibitors are already in clinical trials for cancer treatment (Brown, 1999). The use of inhibitors that effectively block the enzymatic activity of plasmin will probably produce severe side effects (see below), while an attractive approach for inhibition of Plg activation in cancer is blocking of the receptor binding of uPA (Danø *et al.*, 1994; Kim *et al.*, 1998), which will probably be virtually non-toxic (Bugge *et al.*, 1995b). If the massive up-regulation of MMP expression seen upon galardin treatment in this study also occurs during treatment of cancer with MMP inhibitors, this may raise problems as a mechanism for cancer cells to acquire resistance.

The conclusions based on observations on tissue remodeling processes in Plg-deficient mice are probably very relevant also for the human situation. Recently, the rare, but long known and well described, inherited disease ligneous conjunctivitis was linked to Plg deficiency (Mingers *et al.*, 1997; Schuster *et al.*, 1997, 1999; Schott *et al.*, 1998). This disease is characterized by pseudomembranous 'wood-like' lesions of the conjunctiva and other mucous membranes (Bouisson *et al.*, 1847; Bateman *et al.*, 1986; Hydayat *et al.*, 1987; Schuster *et al.*, 1997), and there are many similarities with the phenotype observed in the Plg-deficient mice (Bugge *et al.*, 1995a; Ploplis *et al.*, 1995; Rømer *et al.*, 1996; Drew *et al.*, 1998), including the impaired wound healing which was recently described in two patients with inherited Plg deficiency (Mingers *et al.*, 1997; Schott *et al.*, 1998). Ligneous conjunctivitis patients often display serious symptoms, such as hydrocephalus and blindness, indicating that long-term use of effective plasmin inhibitors for therapeutic purposes, including the treatment of cancer patients, may not be feasible due to severe side effects.

Materials and methods

Tissue preparation

Adult mice were anaesthetized by subcutaneous administration of 0.03 ml/10 g of a 1:1 mixture of Dormicum (midazolam 5 mg/ml) and Hypnorm (fluanison 5 mg/ml and fentanyl 0.1 mg/ml) before surgery. Full-thickness 20 mm long incisional skin wounds were made mid-dorsally with a scalpel. The wounds were neither dressed nor sutured. In this wound model, the formation of a dry scab between the wound edges has the effect that the epidermal edges are kept well separated, despite the initial wound contraction (Rømer *et al.*, 1996). This means that the new epidermal layer is formed entirely by keratinocytes migrating from the wound edges and not from underlying hair follicles. In this model, the significance of skin contraction and the hair growth cycle is minimized during the restoration of an intact epidermal wound cover. The mice were caged individually until sacrifice by perfusion–fixation with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The wound areas were removed, bisected in the mid-transversal plane, fixed in 4% paraformaldehyde overnight (Rømer *et al.*, 1991, 1994) and then paraffin embedded. Sections were cut perpendicular to the longitudinal direction of the wounds. Animal care at The Department of Experimental Medicine, University of Copenhagen and Rigshospitalet, Copenhagen, Denmark was in accordance with the national guidelines. There were no gross or microscopic signs of infections of the wounds or mice during the course of any of the experiments.

Animals and animal treatment

Plg gene-targeted mice of a mixed 129/Black Swiss background (Bugge *et al.*, 1995) were backcrossed to C57Bl/6J mice for 2–6 generations and used throughout the study, except for the experiment with mice backcrossed into the NIHS background shown in Figure 2. For all breeding, heterozygous $Plg^{+/-}$ mice were used. In all experiments involving wild-type mice as controls, these were littermates of the Plg-deficient mice. All mice used for experiments were between 7 and 11 weeks old at the start of the experiment. All experimental evaluations were performed by an investigator unaware of animal genotype. Genotyping of the Plg alleles was performed as described (Bugge *et al.*, 1996). The MMP inhibitor *N*-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (galardin) was synthesized as described (Grobelny *et al.*, 1992). Galardin inhibits the enzymatic activity of a number of MMPs, including gelatinase A, gelatinase B, interstitial collagenase, neutrophil collagenase and stromelysin-1 (Grobelny *et al.*, 1992; Levy *et al.*, 1998). Galardin was administered i.p. as a 20 mg/ml slurry in 4% CMC, 0.9% saline. Injections began immediately after wounding and were continued every 24 h thereafter until the end of the experiment. Statistical evaluation of differences between groups of mice in the time taken to complete wound healing was done with the log rank test.

Computer-assisted morphometry

Keratinocyte migration was measured microscopically on tissue sections stained immunohistochemically with anti-keratin IgG (see below). The length of the epidermal tongue was measured from the tip of the leading edge keratinocytes to the point in the zone of proliferation, where a shift from two to three layers of keratinocytes was identified. Indication of this point in the proliferation zone and the tip of the epidermal wedge, and the drawing of a line along the base of the tongue between these sites by image analysis (NIH Image/ppc 1.56b30 software for Macintosh) were performed by an observer unaware of the genotype and treatment of the mice. The mean distance of migration in each group was calculated as an estimated mean, and the standard error of the mean (SEM) as the standard deviation divided by the square root of the number of animals in the group.

Immunohistochemistry

Tissue sections were stained immunohistochemically by the peroxidase–anti-peroxidase method (Rømer *et al.*, 1991) with rabbit polyclonal antibodies directed against mouse keratin (Cappel, Organon Teknika, PA) or mouse fibrinogen (Bugge *et al.*, 1995a), fibronectin (Dako, Denmark) or laminin (L9393, Sigma, IL). Each experiment included controls with omission of the primary antibody and substitution of the primary antibody with non-immune rabbit IgG. These were all negative.

In situ hybridization

In situ hybridization was performed on paraffin sections essentially as described (Rømer *et al.*, 1991). ^{35}S-labeled RNA sense and antisense probes were generated by *in vitro* transcription from subclones of the following mouse cDNA fragments: gelatinase A (604–1165) in pSP64 and pSP65 or (1924–2259) in pGEM-3 (Reponen *et al.*, 1992); gelatinase B (779–1073) or (1918–2239) in pSP64 and pSP65 (Reponen *et al.*, 1994); and macrophage metalloelastase (900–1250) in T3T7-19 (Shapiro *et al.*, 1992). From stromelysin-1 cDNA (Hammani *et al.*, 1992) fragments of (2205–2918) and (3115–4051); from stromelysin-3 cDNA (Lefebvre *et al.*, 1992) a fragment of (1–996); and from collagenase-3 cDNA (Henriet *et al.*, 1992), the two *Eco*RI fragments of 485 and 811 bp were subcloned in pBluescript KS+. The mouse MT-1 MMP fragment (1122–1867, DDBJ/EMBL/GenBank accession No. X83536) was generated by PCR amplification of reverse-transcribed mRNA extracted from involuting mammary gland tissue with the primers: 5′ACATCTGTGACGGGAACTTTGACA and 5′ATGGCGTCTGAAG-AAGAAGACAGCGAG and subcloned in pBluescript KS+. Expression of mRNA for gelatinase A, gelatinase B, stromelysin-1 and collagenase-3 was detected in some of the experiments with two non-overlapping probes. In these cases, identical results were obtained. Sense probes were included in all experiments and in all cases gave negative results.

Acknowledgements

We thank Dr Karl Tryggvason for gelatinase-A and -B cDNA, Dr Yves Eeckhout for stromelysin-1 and collagenase-3 cDNA, Dr Paul Basset for stromelysin-3 cDNA, and Dr Steven D.Shapiro for macrophage metalloelastase cDNA. We are grateful to Dr Lars Bo Hansen for synthesis of galardin, and to Britt Nagel Ebstrup, Kirsten Lund Jacobsen and Pia Gottrup Knudsen for excellent technical assistance. This work was supported financially by the Danish Biotechnology Program, Center

for Medical Biotechnology, the Danish Cancer Society, the Danish Medical Research Council, the Sofus Friis Foundation, the Vera and Carl Johan Michaelsen Foundation and the Danish Cancer Research Foundation.

References

Bateman,J.B., Pettit,T.H., Isenberg,S.J. and Simons,K.B. (1986) Ligneous conjunctivitis: an autosomal recessive disorder. *J. Pediatr. Ophthalmol. Strabismus*, **23**, 137–140.

Bini,A., Itoh,Y., Kudryk,B.J. and Nagase,H. (1996) Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly404–Ala405 peptide bond. *Biochemistry*, **35**, 13056–13063.

Blasi,F. (1993) Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. *BioEssays*, **15**, 105–111.

Bouisson,M. (1847) Ophthalmie sur-aigue avec formation de pseudomembranes à la surface de la conjonctive. *Ann. Oculist.*, **17**, 100–104.

Brown,P.D. (1999) Clinical studies with matrix metalloproteinase inhibitors. *Acta Pathol. Microbiol. Scand.*, **107**, 174–180.

Bugge,T.H., Flick,M.J., Daugherty,C.C. and Degen,J.L. (1995a) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev.*, **9**, 794–807.

Bugge,T.H., Suh,T.T., Flick,M.J., Daugherty,C.C., Rømer,J., Solberg,H., Ellis,V., Danø,K. and Degen,J.L. (1995b) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J. Biol. Chem.*, **270**, 16886–16894.

Bugge,T.H., Kombrinck,K.W., Flick,M.J., Daugherty,C.C., Danton,M.J. and Degen,J.L. (1996) Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell*, **87**, 709–719.

Bugge,T.H., Kombrinck,K.W., Xiao,Q., Holmback,K., Daugherty,C.C., Witte,D.P. and Degen,J.L. (1997) Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. *Blood*, **90**, 4522–4531.

Bugge,T.H. *et al.* (1998) Reduced metastasis of polyoma virus middle T antigen-induced mammary cancer in plasminogen-deficient mice. *Oncogene*, **16**, 3097–3104.

Chen,Z.L. and Strickland,S. (1998) Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. *Cell*, **91**, 917–925.

Danø,K., Andreasen,P.A., Grøndahl Hansen,J., Kristensen,P., Nielsen,L.S. and Skriver,L. (1985) Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.*, **44**, 139–266.

Danø,K., Behrendt,N., Brünner,N., Ellis,V., Ploug,M. and Pyke,C. (1994) The urokinase receptor: protein structure and role in plasminogen activation and cancer invasion. *Fibrinolysis*, **8**, 189–203.

Drew,A.F., Kaufman,A.H., Kombrinck,K.W., Danton,M.J., Daugherty,C.C., Degen,J.L. and Bugge,T.H. (1998) Ligneous conjunctivitis in plasminogen-deficient mice. *Blood*, **91**, 1616–1624.

Dvorak,H.F. (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.*, **315**, 1650–1659.

Edwards,D.R. and Murphy,C. (1998) Proteases—invasion and more. *Nature*, **394**, 527–528.

Galardy,R.E., Grobelny,D., Foellmer,H.G. and Fernandez,L.A. (1994) Inhibition of angiogenesis by the matrix metalloprotease inhibitor *N*-L-tryptophan methylamide. *Cancer Res.*, **54**, 4715–4718.

Gijbels,K., Galardy,R.E. and Steinman,L. (1994) Reversal of experimental autoimmune encephalomyelitis with a hydroxamate inhibitor of matrix metalloproteases. *J. Clin. Invest.*, **94**, 2177–2182.

Grobelny,D., Poncz,L. and Galardy,R.E. (1992) Inhibition of human skin fibroblast collagenase, thermolysin and *Pseudomonas aeruginosa* elastase by peptide hydroxamic acids. *Biochemistry*, **31**, 7152–7154.

Grøndahl-Hansen,J., Lund,L.R., Ralfkiær,E., Ottevanger,V. and Danø,K. (1988) Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelialization *in vivo*. *J. Invest. Dermatol.*, **90**, 790–795.

Guo,L., Degenstein,L. and Fuchs,E. (1996) Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.*, **10**, 165–175.

Hammani,K., Henriet,P. and Eeckhout,Y. (1992) Cloning and sequencing of a cDNA encoding mouse stromelysin 1. *Gene*, **120**, 321–322.

Henriet,P., Rousseau,G.G. and Eeckhout,Y. (1992) Cloning and sequencing of mouse collagenase cDNA. Divergence of mouse and rat collagenases from the other mammalian collagenases. *FEBS Lett.*, **310**, 175–178.

Hewitt,R. and Danø,K. (1996) Stromal cell expression of components of matrix-degrading protease systems in human cancer. *Enzyme Protein*, **49**, 163–173.

Hiraoka,N., Allen,E., Apel,I.J., Gyetko,M.R. and Weiss,S.J. (1998) Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell*, **95**, 365–377.

Hydayat,A.A. and Riddle,P.J. (1987) Ligneous conjunctivitis. A clinicopathologic study of 17 cases. *Ophthalmology*, **94**, 949–959.

Johnsen,M.J., Lund,L.R., Rømer,J., Almholt,K. and Danø,K. (1998) Cancer invasion and tissue remodeling. Common themes in proteolytic matrix degradation. *Curr. Opin. Cell Biol.*, **1**, 667–671.

Kim,J., Yu,W., Kovalski,K. and Ossowski,L. (1998) Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell*, **94**, 353–362.

Lefebvre,O., Wolf,C., Limacher,J.M., Hutin,P., Wendling,C., LeMeur,M., Basset,P. and Rio,M.C. (1992) The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. *J. Cell Biol.*, **119**, 997–1002.

Levy,D.E. *et al.* (1998) Matrix metalloproteinase inhibitors: a structure-activity study. *J. Med. Chem.*, **41**, 199–223.

Liotta,L.A., Steeg,P.S. and Stetler-Stevenson,W.G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, **64**, 327–336.

Lund,L.R., Rømer,J., Thomasset,N., Solberg,H., Pyke,C., Bissell,M.J., Danø,K. and Werb,Z. (1996) Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development*, **122**, 181–193.

Mignatti,P. and Rifkin,D.B. (1996) Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme Protein*, **49**, 117–137.

Mingers,A.M., Heimburger,N., Zeller,P., Kreth,H.W. and Schuster,V. (1997) Homozygous type 1 plasminogen deficiency. *Semin. Thromb. Hemostasis*, **23**, 259–264.

Moss,M.L. *et al.* (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature*, **385**, 733–736.

Mazzieri,R., Masiero,L., Zanetta,L., Monea,S., Onisto,M., Garbisa,S. and Mignatti,P. (1997) Control of type IV collagenase activity by components of the urokinase–plasmin system: a regulatory mechanism with cell-bound reactants. *EMBO J.*, **16**, 2319–2332.

Murphy,G., Ward,R., Gavrilovic,J. and Atkinson,S. (1992) Physiological mechanisms for metalloproteinase activation. *Matrix* (Suppl.), **1**, 224–230.

Pepper,M.S., Vassalli,J.D., Montesano,R. and Orci,L. (1987) Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol.*, **105**, 2535–2541.

Peschon,J.J. *et al.* (1998) An essential role for extodomain shedding in mammalian development. *Science*, **282**, 1281–1284.

Ploplis,V.A., Carmeliet,P., Vazirzadeh,S., Van,V.I., Moons,L., Plow,E.F. and Collen,D. (1995) Effects of disruption of the plasminogen gene on thrombosis, growth and health in mice. *Circulation*, **92**, 2585–2593.

Reponen,P., Sahlberg,C., Huhtala,P., Hurskainen,T., Thesleff,I. and Tryggvason,K. (1992) Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. *J. Biol. Chem.*, **267**, 7856–7862.

Reponen,P., Sahlberg,C., Munaut,C., Thesleff,I. and Tryggvason,K. (1994) High expression of 92-kDa type IV collagenase (gelatinase) in the osteoclast lineage during mouse development. *Ann. NY Acad. Sci.*, **732**, 472–475.

Rinkenberger,J.L., Cross,J.C. and Werb,Z. (1997) Molecular genetics of implantation in the mouse. *Dev. Genet.*, **2**, 6–20.

Rømer,J., Lund,L.R., Eriksen,J., Ralfkiær,E., Zeheb,R., Gelehrter,T.D., Danø,K. and Kristensen,P. (1991) Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. *J. Invest. Dermatol.*, **97**, 803–811.

Rømer,J., Lund,L.R., Eriksen,J., Pyke,C., Kristensen,P. and Danø,K. (1994) The receptor for urokinase-type plasminogen activator is expressed by keratinocytes at the leading edge during re-epithelialization of mouse skin wounds. *J. Invest. Dermatol.*, **102**, 519–522.

Rømer,J., Bugge,T.H., Pyke,C., Lund,L.R., Flick,M.J., Degen,J.L. and Danø,K. (1996) Impaired wound healing in mice with a disrupted plasminogen gene. *Nature Med.*, **2**, 287–292.

Sappino,A.P., Huarte,J., Belin,D. and Vassalli,J.D. (1989) Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. *J. Cell Biol.*, **109**, 2471–2479.

Schott,D. *et al.* (1998) Successful replacement therapy with a purified plasminogen concentrate in an infant with ligneous conjunctivitis

and homozygous plasminogen deficiency. *N. Engl. J. Med.*, **339**, 1679–1686.

Schuster,V., Mingers,A.M., Seidenspinner,S., Nussgens,Z., Pukrop,T. and Kreth,H.W. (1997) Homozygous mutations in the plasminogen gene of two unrelated girls with ligneous conjunctivitis. *Blood*, **90**, 958–966.

Schuster,V. *et al.* (1999) Compound-heterozygous mutations in the plasminogen gene predispose to the development of ligneous conjunctivitis. *Blood*, **93**, 3457–3466.

Shapiro,S.D., Griffin,G.L., Gilbert,D.J., Jenkins,N.A., Copeland,N.G., Welgus,H.G., Senior,R.M. and Ley,T.J. (1992) Molecular cloning, chromosomal localization and bacterial expression of a murine macrophage metalloelastase. *J. Biol. Chem.*, **267**, 4664–4671.

Shrivastava,A. *et al.* (1997) An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol. Cell*, **1**, 25–34.

Solorzano,C.C. *et al.* (1997) A matrix metalloproteinase inhibitor prevents processing of tumor necrosis factor alpha (TNF α) and abrogates endotoxin-induced lethality. *Shock*, **7**, 427–431.

Sternlicht,M.D. and Werb,Z. (1999) Extracellular matrix proteinases. In Kreis,T. and Vale,R. (eds), *Guidebook to the Extracellular Matrix and Adhesion Proteins*. Oxford University Press, New York, in press.

Strauss,B.H. *et al.* (1996) *In vivo* collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circ. Res.*, **79**, 541–550.

Talhouk,R.S., Bissell,M.J. and Werb,Z. (1992) Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell. Biol.*, **118**, 1271–1282.

Teesalu,T., Blasi,F. and Talarico,D. (1998) Expression and function of the urokinase type plasminogen activator during mouse hemochorial placental development. *Dev. Dynam.*, **213**, 27–38.

Tsirka,S.E., Bugge,T.H., Degen,J.L. and Strickland,S. (1997) Neuronal death in the central nervous system demonstrates a non-fibrin substrate for plasmin. *Proc. Natl Acad. Sci. USA*, **94**, 9779–9781.

Vogel,W., Gish,G.D., Alves,F. and Pawson,T. (1997) The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol. Cell*, **1**, 13–23.

Waltz,D.A., Natkin,L.R., Fujita,R.M., Wei,Y. and Chapman,H.A. (1997) Plasmin and plasminogen-activator inhibitor type-1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. *J. Clin. Invest.*, **100**, 58–67.

Werb,Z. (1997) ECM and cell surface proteolysis: regulating cellular ecology. *Cell*, **91**, 439–442.

Whitelock,J.M., Murdoch,A.D., Iozzo,R.V. and Underwood,P.A. (1996) The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth-factor by stromelysin, collagenase, plasmin and heparanases. *J. Biol. Chem.*, **271**, 10079–10086.

Witte,M.B., Thornton,F.J., Kiyama,T., Efron,D.T., Schulz,G.S., Moldawer,L.L. and Barbul,A. (1998) Metalloproteinase inhibitors and wound healing: a novel enhancer of wound strength. *Surgery*, **124**, 464–470.

Wolfsberg,T.G., Primakoff,P., Myles,D.G. and White,J.M. (1995) ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain: multipotential functions in cell–cell and cell–matrix interactions. *J. Cell Biol.*, **131**, 275–278.

Received January 7, 1999; revised and accepted July 5, 1999

Exhibit F

1: Eur J Cell Biol. 1997 Oct;74(2):111-22.

Tissue inhibitors of metalloproteinases: structure, regulation and biological functions.

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP.

Department of Science and Technology, Quilmes National University, Buenos Aires/Argentina.

Four members of the tissue inhibitor of metalloproteinases (TIMP) family have been characterized so far, designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMP-1 and TIMP-2 are capable of inhibiting the activities of all known matrix metalloproteinases (MMPs) and as such play a key role in maintaining the balance between extracellular matrix (ECM) deposition and degradation in different physiological processes. Accelerated breakdown of ECM occurs in various pathological processes, including inflammation, chronic degenerative diseases and tumor invasion. TIMP-1 and TIMP-2 can inhibit tumor growth, invasion, and metastasis in experimental models which has been associated with their MMP inhibitory activity. Recent developments in TIMP research suggest that TIMP-1 and TIMP-2 are multifunctional proteins with diverse actions. Both inhibitors exhibit growth factor-like activity and can inhibit angiogenesis. Structure-function studies have separated the MMP inhibitory activity of TIMP-1 from its growth promoting effect. TIMP-1 has also been implicated in gonadal steroidogenesis and as a cellular elongation factor. TIMP-3 is the only member of the TIMP family which is found exclusively in the extracellular matrix (ECM). It is regulated in a cell cycle-dependent fashion in certain cell types and may serve as a marker for terminal differentiation. The most recently discovered TIMP, TIMP-4, may function in a tissue-specific fashion in extracellular matrix hemostasis. The main aim of this article is to review recent literature on TIMPs with special emphasis on their biological activities and the possibility that they may have paradoxical roles in tumor progression.

Publication Types:
- Review

- Review, Tutorial

PMID: 9352216 [PubMed - indexed for MEDLINE]

Exhibit G

1: Adv Ren Replace Ther. 1998 Jul;5(3):205-11.

Peritoneal fluid markers of mesothelial cells and function.

Ho-dac-Pannekeet MM.

Department of Nephrology, Academic Medical Center, Amsterdam, The Netherlands.

Peritoneal structural changes are likely to result in functional deterioration of the peritoneal membrane. For the purpose of early detection of these changes, markers of mesothelial cells that can be measured in peritoneal effluent could provide easily accessible information in individual peritoneal dialysis (PD) patients. In this review, current knowledge on a number of these markers is summarized, such as cancer antigen (CA) 125, phospholipids, hyaluronan, and factors involved in the coagulation system. Although only analyzed in limited studies so far, this approach to understanding changes in the peritoneal membrane seems to be valid and warrants further evaluation in the future, especially in combination with functional studies and peritoneal biopsies.

Publication Types:
- Review
- Review, Tutorial

PMID: 9686631 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

Exhibit H

1: Perit Dial Int. 1999;19 Suppl 2:S212-6.

A simple and fast method to estimate peritoneal membrane transport characteristics using dialysate sodium concentration.

Wang T, Waniewski J, Heimburger O, Bergstrom J, Werynski A, Lindholm B.

Department of Clinical Sciences, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden.

BACKGROUND: The peritoneal equilibration test (PET) is widely used to classify a patient's peritoneal transport characteristics. However, PET is laborious and the prediction of fluid removal based on PET is generally poor. It is believed that osmosis by glucose occurs partially through transcellular water channels, resulting in sieving of sodium and decrease of dialysate sodium concentration when using hypertonic glucose dialysate. OBJECTIVE: In this study, we investigated the possibility of using dialysate sodium concentration to classify the patient's peritoneal transport characteristics. METHODS: A 6-hour dwell study with frequent dialysate and plasma sampling was performed in 46 patients using 2 L of 3.86% glucose dialysate with 131I-albumin as an intraperitoneal volume (IPV) marker. The peritoneal transport of sodium, creatinine, glucose, and fluid was evaluated. RESULTS: The dialysate sodium concentration at 240 min (D(Na240)) significantly correlated with D/P creatinine ($r = 0.76$, $p < 0.001$) and D/D0 glucose ($r = -0.83$, $p < 0.001$) at 240 min of the dwell (better than dialysate sodium concentration at any other time of the dwell). DNa240 also significantly correlated with IPV at 240 min of the dwell ($r = -0.61$, $p < 0.001$)(better than D/P creatinine and D/D0 glucose). There were significant correlations between D(Na240) and the sodium-sieving coefficient ($r = 0.71$, $p < 0.001$) and the diffusive mass transfer coefficient for sodium ($r = 0.50$, $p < 0.001$). When using D(Na240) to divide the patients into four groups, as in the PET method, no significant difference was found between the two methods. CONCLUSION: Using 3.86% glucose solution, D(Na240) can be used instead of D/P creatinine to classify patients into different transport groups. D(Na240) provides a better prediction of peritoneal fluid transport and reflects both the diffusive and convective transport properties of the membrane. As only one dialysate sample (and no blood

sample) is needed, D(Na240) may offer important clinical advantages compared with PET.

PMID: 10406521 [PubMed - indexed for MEDLINE]

Exhibit I

FERTILITY AND STERILITY®
VOL. 73, NO. 3, MARCH 2000

Published by Elsevier Science Inc.
Printed on acid-free paper in U.S.A.

Matrix metalloproteinases and their tissue inhibitors in antegradely shed menstruum and peritoneal fluid

Carolien A. M. Koks, M.D., Patrick G. Groothuis, Ph.D.,† Peronneke Slaats, M.S.,‡ Gerard A. J. Dunselman, M.D.,* Anton F. P. M. de Goeij, Ph.D.,† and Johannes L. H. Evers, M.D.**

Academisch Ziekenhuis Maastricht, Maastricht University, Maastricht, the Netherlands

Objective: To investigate the expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in antegradely shed menstruum and peritoneal fluid.

Design: A cell biological and immunohistochemical study.

Setting: Tertiary care university medical center.

Intervention(s): Immunohistochemistry was performed on cryostat sections and cultures of menstrual endometrium. Zymography was used to characterize MMP activity in peritoneal fluid, in menstrual serum, and in conditioned medium. Western blot analysis was used to further identify the MMPs in these fluids.

Main Outcome Measure(s): Staining of MMPs and TIMPs in cryostat sections and cultures and MMP expression and activity in peritoneal fluid and menstrual blood serum.

Result(s): Strong staining for MMP-1 and MMP-3 was observed in stroma and for MMP-7 in epithelium. Matrix metalloproteinase-2 and MMP-9 were weakly expressed in stroma. Both TIMP-1 and TIMP-2 were expressed in menstrual endometrium. Menstrual serum showed a pattern of MMP activity on zymography different from peritoneal fluid. Western blot analysis showed the presence of MMP-7 and MMP-9 in menstrual serum.

Conclusion(s): Antegradely shed menstrual endometrium expresses several MMPs and TIMPs, even after culturing for 24 hours. MMP activity in menstrual serum is different from and more intense than MMP activity in peritoneal fluid. These enzymes may be involved in the early invasion of menstrual endometrium into the extracellular matrix of the peritoneum. (Fertil Steril® 2000;73:604–12. ©2000 by American Society for Reproductive Medicine.)

Key Words: Menstrual fluid, zymography, Western blotting, immunohistochemistry, endometriosis

Received February 22, 1999; revised and accepted October 18, 1999.

Reprint requests: Carolien A.M. Koks, M.D., Department of Obstetrics and Gynecology, Academisch Ziekenhuis Maastricht, P.O. Box 5800, 6202AZ Maastricht, the Netherlands (FAX: +31-433874765; E-mail: jacques_carolien.yahoo.com).

* Department of Obstetrics and Gynecology.

† Department of Pathology.

‡ Department of Medicine.

0015-0282/00/$20.00
PII S0015-0282(99)00566-X

Matrix metalloproteinases (MMPs) are highly homologous proteolytic zinc enzymes responsible for degradation of extracellular matrix components such as collagen, proteoglycans, fibronectin, and laminin (1). The balance between MMPs and their tissue inhibitors (TIMPs) plays an important role in normal physiologic events, such as tissue repair, embryogenesis, extravillous trophoblast invasion, and menstruation (2–5), and in pathologic processes, such as rheumatoid arthritis and tumor invasion (6).

Studies on MMP expression during the menstrual cycle have shown that MMPs are mostly expressed during menstruation when extensive remodeling of endometrium occurs (5, 7–10). Matrix metalloproteinases are not only produced by endometrial stroma and epithelium but also by polymorphic mononuclear leukocytes (PMNs) (10, 11–14).

The regulation of endometrial MMPs at the time of menstruation is multifactorial. Progesterone withdrawal, however, is obligatory for MMP production (7–9, 14–16). At the onset of menstruation there is an influx of neutrophils, macrophages, and eosinophils in endometrium. These cells not only produce mediators that upregulate MMP expression but also provide a new source of specific MMPs (10–13). The principal MMPs involved in the process of menstruation remain to be determined.

During menstruation the endometrial tissue is shed both antegradely and retrogradely. Ret-

rograde menstruation is considered to be pivotal in the development of endometriosis. In a previous study we showed that the concentration of aminoterminal propeptide of type III procollagen in peritoneal fluid (PF) is increased in patients with early endometriosis compared with controls, suggesting an active invasion process (17). Treatment of endometrium with TIMP-1 in vitro followed by intraperitoneal injections of TIMP-1 reduced the development of ectopic lesions in nude mice injected intraperitoneally with human endometrial tissue (18). Therefore, local disturbance of the MMP-TIMP equilibrium may increase the invasive behavior of shed endometrial cells and may ultimately lead to the development of endometriosis.

The purpose of the present study was to investigate the expression of MMPs and TIMPs in naturally shed menstrual endometrium directly after shedding and after in vitro culture for 24 hours. To determine whether MMP activity in the PF originates from menstruum or from other constituents of the PF, we compared MMP activity in menstrual serum with MMP activity in PF.

To this end, immunohistochemistry was performed on cryostat sections and cultures of menstrual endometrial tissue, and MMP activity was characterized in menstrual serum, PF, and conditioned medium by zymography and Western blotting.

MATERIALS AND METHODS

Characteristics of Volunteers

Menstruum was collected by 12 volunteers with regular menstrual cycles varying from 25 to 34 days with 4–7 days of blood loss. None of the volunteers used oral anticonceptives or intrauterine copper devices. Peritoneal fluid was collected from 11 patients undergoing laparoscopy for sterilization (n = 5) or infertility work-up (n = 6). All gave written informed consent. The institutional review board and the medical ethics committee approved the study protocol.

Menstrual Effluent Collection

Menstrual effluent was collected with a menstrual cup for 2–3 hours during the 1st, 2nd, and 3rd day of the menstrual period. Day one was defined as the 24 hours from the first awareness of menstrual bleeding onward. The menstrual cup was a soft natural rubber cup (Keeper, Den Haag, the Netherlands) that has been used in our previous studies (19, 20). After collection, the menstrual effluent was transferred into a plastic container and delivered immediately to the laboratory.

Menstruum Preparations

The menstrual effluent samples were centrifugated at 1,800 × *g* for 10 minutes. The supernatant (serum) was collected, frozen immediately in isopentane in dry ice, and stored at −80°C until analyzed. The pellet was washed with phosphate-buffered saline (PBS) and layered on a Ficoll-Paque gradient to remove red blood cells. After centrifugation for 5 minutes at 2,500 × *g*, menstrual tissue was collected from the interface. The endometrial tissue was frozen immediately in isopentane immersed in dry ice and stored at −80°C until analyzed.

Endometrial tissue used for cultures was isolated as described above, but washes were performed with complete culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM)-F-12 (Life Technologies BV, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamin, and penicillin 100 U/mL, and streptomycin 100 μg/mL (Life Technologies BV) instead of PBS. After the Ficoll gradient separation, menstrual tissue was washed once, resuspended in culture medium, filtered through a 100-μm nylon filter (Micronic, Lelystad, the Netherlands) and a 30-μm polyamid filter (Stokvis and Smits, Ijmuiden, the Netherlands). The suspension filtered through the 30-μm filter was layered on a Ficoll-Paque gradient to remove red blood cells and to obtain small tissue fragments and single cells.

The tissue fragments retained on the 100- and 30-μm filter and the tissue of <30 μm collected from the interface of the Ficoll-Paque gradient was pooled and resuspended in culture medium.

Peritoneal Fluid Collection

Peritoneal fluid samples were obtained from women undergoing diagnostic laparoscopy for infertility workup (n = 6) or for sterilization (n = 5). Collections were approved by the hospital's medical ethics committee, and written informed consent was obtained before operation. All women who underwent a laparoscopy for infertility work-up had ovulatory cycles, as confirmed by ultrasonography or an adequate rise in serum progesterone in the luteal phase of the cycle. The women undergoing laparoscopy for sterilization had regular cycles and proved fertility. None of them (11) had visible endometriosis.

The fluid was collected immediately after introduction of the second trocart. The collected fluid was transported to the laboratory on ice and centrifuged immediately for 3 minutes. The pellet was discarded, and the supernatant was divided in small aliquots and frozen immediately in isopentane in dry ice and stored at −80°C until use. Peritoneal fluid samples were collected during cycle days 3, 4, 7, 10, 11, 12, 13, 15, 20, 22, and 26.

Cultures of Endometrial Tissue for Immunohistochemistry

Endometrial fragments retained on the 100- and 30-μm filters and on the Ficoll-Paque after filtration were cultured in culture medium on uncoated glass slides. Cultures were performed during 24 hours at 37°C. To a part of the cultures 5 μM of monensin (Sigma, St. Louis, MO) was added 4–16 hours before fixation. This ionophore blocks excretion of proteins from the Golgi apparatus but does not block their

TABLE 1

Primary antibodies used for immunohistochemistry.

Antigen	Antibody	Dilution HC	WB	Source
MMP-1	Ab 1; clone; 41-1ES epitope: A2 332-350	1:10	2.5μg/mL	Oncogene Research Products, Cambridge, MA
MMP-2	Ab 3; clone; 42-5001 epitope: A2 524-539	1:10	2.5μg/mL	Oncogene Research Products
MMP-3	Ab 1; clone; 55-2A4 epitope: ND	1:10	2.5μg/mL	Oncogene Research Products
MMP-7	clone 141-7b2 epitope: ND	1:100	1μg/mL	Fuji Chemical Industries LTD, Toyama, Japan
MMP-9	Ab 1; clone; 6-613 antibody 1 epitope: ND	1:10	2.5μg/mL	Oncogene Research Products
MMP-9	Ab 2; clone; 7-11 epitope: ND	1:10	2.5μg/mL	Oncogene Research Products
TIMP-1	Ab 1; clone: 7-6C1 epitope: ND	1:10	2.5μg/mL	Oncogene Research Products
TIMP-2	Ab 1; clone: T2-101 epitope: ND	1:20	2.5μg/mL	Oncogene Research Products

HC = immunohistochemistry; WB = Western blot; Ab = antibody; ND = not determined.

Koks. MMPs and TIMPs. Fertil Steril 2000.

production (21). After culturing, the glass slides were rinsed in PBS and fixed immediately in methanol at −20°C for 1 minute followed by an acetone dip at −20°C.

Preparation of Conditioned Medium

The obtained menstrual endometrial tissue from four volunteers were cultured in plastic petri dishes with 5 mL of culture medium. After culturing for 24 hours the medium was collected, centrifuged, and the supernatant was immediately frozen in isopentane immersed in dry ice and stored at −80°C until use.

Immunohistochemical Staining

Cryostat sections were cut at 4–6 μm and mounted on slides. The sections were air dried and fixed with methanol at −20°C for 1 minute followed by an acetone dip at −20°C. A total of 12 menstrual endometrial cryostat samples and 4–8 endometrial cell cultures were used for immunohistochemistry with each of the various antibodies. Slides were washed three times for 5 minutes in PBS and immersed in 3% hydrogen peroxide and 0.01% sodium azide to block endogenous peroxidase activity. The sections were washed again three times in PBS.

Monoclonal antibodies against MMP-1, -2, -3, -7, MMP-9 (2), and TIMP-1 and -2 were used (Table 1). Except for MMP-7 and MMP-9 antibody 2, the antibodies recognized latent and active MMP.

Incubation with the primary antibodies, appropriately diluted in PBS containing 1% bovine serum albumin (BSA), was performed overnight at 4°C. After being washed three times for 10 minutes in PBS, the samples were incubated for 30 minutes at room temperature with rabbit anti-mouse immunoglobulin (Ig)G conjugated with horseradish peroxidase (dilution 1:100; Dako A/S, Glostrup, Denmark). After being washed in PBS, antibody binding was visualized with use of 3,3′-diaminobenzidine (DAB) and 0.3% hydrogen peroxide. Except for MMP-7, imidazol was added to the DAB solution. The slides were counterstained with hematoxylin, dehydrated through alcohols, cleared in xylene, and mounted in Entellan for light microscopy.

Endometrium of cycle day 2 was used as a positive control. Negative controls included sections without the primary antibody, using PBS-BSA 1% instead.

Zymography

Proteinase activity was analyzed by zymography on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels containing gelatin 1 mg/mL or casein 0.5 mg/mL under nonreducing conditions as described by Fisher et al. (22). Conditioned medium (1:4), menstrual serum (1:100), and PF (1:100) samples were diluted with deionized water, for gelatin zymography. For casein zymography the dilutions were 1:100 for PF and menstrual serum and 1:2 for conditioned medium. To control for possible exogenous proteinase contamination, culture medium was also applied on the gel. For semiquantitation, the volume loaded was adjusted according to protein concentration as measured by a protein assay (Bio-Rad Laboratories, North Ryde, Australia). Samples containing 4.5 μg of protein (approximately 10 μL) were loaded on the gels. A protein marker, containing 13 protein standards covering the molecular mass range from 2.3 to 212 kd, was used for size determination (Biolabs, New England, Boston, MA). The gels were run for approximately 2 hours at 100 V at room temperature.

After electrophoresis, the gels were washed two times for 30 minutes at room temperature on a moving platform in 2.5% Triton-X100. The gels were then incubated overnight at 37°C in a shaking water bath in a substrate buffer. After incubation, the gels were stained with Coomassie brilliant blue R250, 0.2% in 4% methanol, and 10% acetic acid in water for 2 hours, and destained in 4% methanol with 10% acetic acid in water. Proteolytic activity appeared as clear bands on a blue background.

TABLE 2

Immunohistochemical staining on cryostat sections of shed endometrium.

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9 Ab-1	MMP-9 Ab-2	TIMP-1	TIMP-2
Stroma	12*	11	12	8	12	11	12	12
Epithelium	6	0	0	12	0	0	8	0
Nuclear staining	3	0	0	0	9	0	1	1

*Number of cases with positive staining over 12 cases tested.

Koks. MMPs and TIMPs. Fertil Steril 2000.

Western Blot Analysis

Menstrual serum and PF samples diluted 1:100 with deionized water and undiluted conditioned medium were mixed with sample buffer (4:1). Samples containing 9 μg of protein (approximately 20 μL) and a prestained protein marker were subjected to SDS–polyacrylamide gel electrophoresis on 10% gels under reducing conditions, and the proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). These filters were blocked overnight at 4°C in 3% nonfat powdered milk in Tris-buffered saline and Tween 20 (TBST).

After blocking, the nitrocellulose filters were washed twice in TBST for 10 minutes. They were incubated overnight at 4°C with monoclonal antibodies against human MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, or TIMP-2 in the recommended concentrations (Table 1). After incubation, the nitrocellulose filters were washed again two to three times for 10 minutes in TBST, and the primary antibodies were complexed with a rabbit anti-mouse IgG alkaline phosphatase conjugate (1:1,000) for 60 minutes at room temperature. After washing the filters for three times during 10 minutes with TBST, the blot was developed by adding the alkaline phosphatase substrate solution (Promega, Madison, WI). As soon as the bands developed to the desired intensity, the reaction was stopped by washing the blot in a solution consisting of 20 mM Tris-HCL and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. The blots were air dried at room temperature.

RESULTS

Immunohistochemistry on Cryostat Sections

Antegradely shed menstrual endometrium expressed all MMPs and TIMPs investigated (Table 2). Matrix metalloproteinase-1 and MMP-3 (Fig. 1b and d) were strongly, TIMP-1 and 2 (Fig. 1h and i) mildly, and MMP-2 and MMP-9 (Fig. 1c, f, and g) weakly expressed in stroma. Staining for MMP-7 (Fig. 1e) was strong in glandular epithelium. Epithelial cells stained weakly for MMP-1 and TIMP-1. Tissue inhibitor of matrix metalloproteinase-1 was observed at the basal side of many epithelial cells, whereas MMP-1 was observed in only few epithelial cells. Matrix metalloproteinase-7, although most prominent in epithelial cells, also stained diffusely in stroma.

In some samples nuclear staining was observed, which was most prominent for MMP-9 (antibody 1) in 9 of 12 samples (Fig. 1f), for MMP-1 in 3 of 12 samples (Fig. 1b), and observed for TIMP-1 and TIMP-2 in only one sample.

Immunohistochemistry on Cultures

The results are presented in Table 3.

Matrix metalloproteinase-1 was expressed in all cultures, and treatment with monensin increased the intensity of staining (Fig. 2b). Matrix metalloproteinase-2 was expressed in all cultures without monensin and in only one culture treated with monensin (Fig. 2c). Despite the addition of monensin, the expression of MMP-2 remained weak. Addition of monensin was necessary to detect expression of MMP-3 (Fig. 2d). Expression of MMP-7 was seen in one culture with and one culture without monensin. It was present in characteristically packed whorls of large flat epithelial cells (Fig. 2e). Expression of MMP-9 (Ab-1 and Ab-2) was weak and seen in two of nine cultures (Fig. 2f and g).

Tissue inhibitor of matrix metalloproteinase-1 and TIMP-2 were expressed in seven of eight and three of six cultures respectively (Fig. 2h and i). Monensin did not increase the staining intensity of these tissue inhibitors of MMPs.

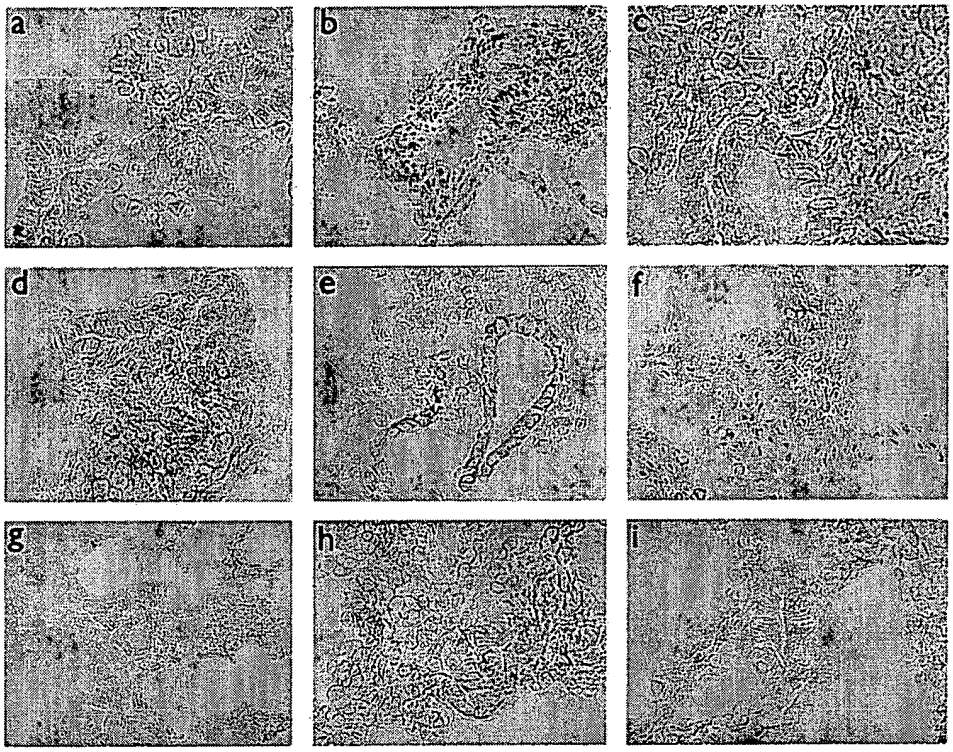
Zymography

Gelatin zymography revealed four major bands for menstrual serum samples (Fig. 3A). One intense band at 92 kd, a second intense band at 67 kd, the third and fourth distinct bands were found at 60 and 52 kd, and some weaker bands were observed. Sometimes a small sharp band was seen at 180 kd. All these bands occurred in the menstrual fluid samples from cycle days 1–3. However, menstrual serum from day 3 showed smaller bands than menstrual serum from the first 2 days of the menstrual cycle.

Conditioned medium revealed three intense bands (67, 60, and 52 kd [Fig. 3A]). A faint band was observed at 92 kd. Normal culture medium (with 10% FCS) also revealed two

FIGURE 1

Immunohistochemical staining on cryostat sections of shed menstrual endometrium (a) control, (b) MMP-1, (c) MMP-2, (d) MMP-3, (e) MMP-7, (f) MMP-9 antibody 1, (g) MMP-9 antibody 2 (h) TIMP-1, and (i) TIMP-2. Original magnification ×100 (g), ×150 (b, f, and i) ×250 (a, c, d, e, and h).



Koks. MMPs and TIMPs. Fertil Steril 2000.

bands, one at 67 kd and the other at 60 kd. These bands were more intense when conditioned medium was used.

Gelatin zymography showed two bands for the PF samples, one strong band at 60 kd and a weaker band at 52 kd (Fig. 3A). There was no difference in pattern and thickness of the bands between PF from different phases of the cycle. In a dilution of 1:10 the PF also showed small bands at 92 and 67 kd, but at this dilution the gel was overloaded for menstrual serum.

Casein zymography of menstrual fluid showed different bands compared with PF (Fig. 3B). Both showed a 92-kd and 67-kd band. The menstrual fluid samples also showed some faint bands at 48, 28, and 19 kd. These bands were not seen in all samples but mainly in menstrual fluid samples from cycle days 1 and 2. Conditioned medium showed a band at 92 kd and one at 52 kd (Fig. 3B). Culture medium (with 10% FCS) revealed one band at 92 kd, one faint band at 67 kd, and some diffuse faint bands between 20–43 kd.

TABLE 3

Immunohistochemistry on cultures of menstrual endometrium with and without addition of monensin.

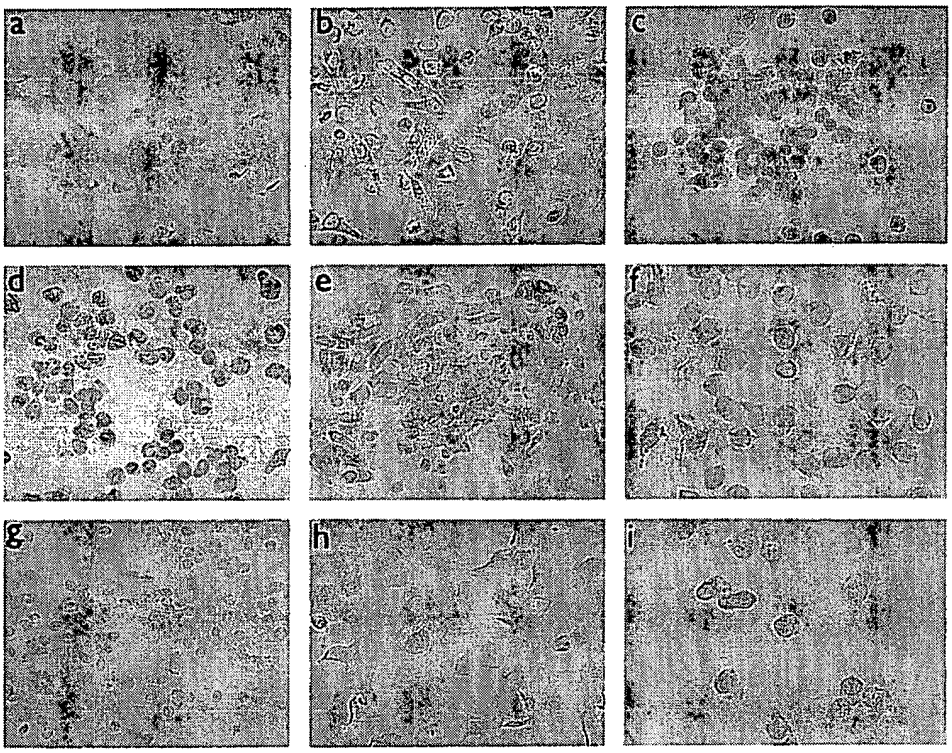
					MMP-9			
	MMP-1	MMP-2	MMP-3	MMP-7	Ab-1	Ab-2	TIMP-1	TIMP-2
All cultures	6/6*	4/7	4/5	2/7	1/4	1/5	7/8	3/6
With monensin	3/3	1/4	4/4	1/3	1/2	0/2	3/4	2/3
Without monensin	3/3	3/3	0/1	1/4	0/2	1/3	4/4	1/3

*Number of cases with positive staining over number of cases tested.

Koks. MMPs and TIMPs. Fertil Steril 2000.

FIGURE 2

Immunohistochemical staining on cultures of shed endometrium (a) control, (b) MMP-1, (c) MMP-2, (d) MMP-3, (e) MMP-7, (f) MMP-9 antibody 1, (g) MMP-9 antibody 2, (h) TIMP-1, and (i) TIMP-2. Original magnification ×100 (a and g), ×150 (c and e), ×250 (b, d, f, h, and i).



Koks. MMPs and TIMPs. Fertil Steril 2000.

With PF, casein zymography showed 92-kd and 67-kd bands that were also seen on gelatin zymography at a dilution of 1:10 (Fig. 3B).

Western Blot

In menstrual serum collected at cycle days 1, 2, and 3 only MMP-7 and MMP-9(Ab-1) showed clear bands exclusively at the expected molecular weights (Fig. 4a and b). For the other MMPs, bands at the expected molecular weight and multiple other faint bands were observed.

DISCUSSION

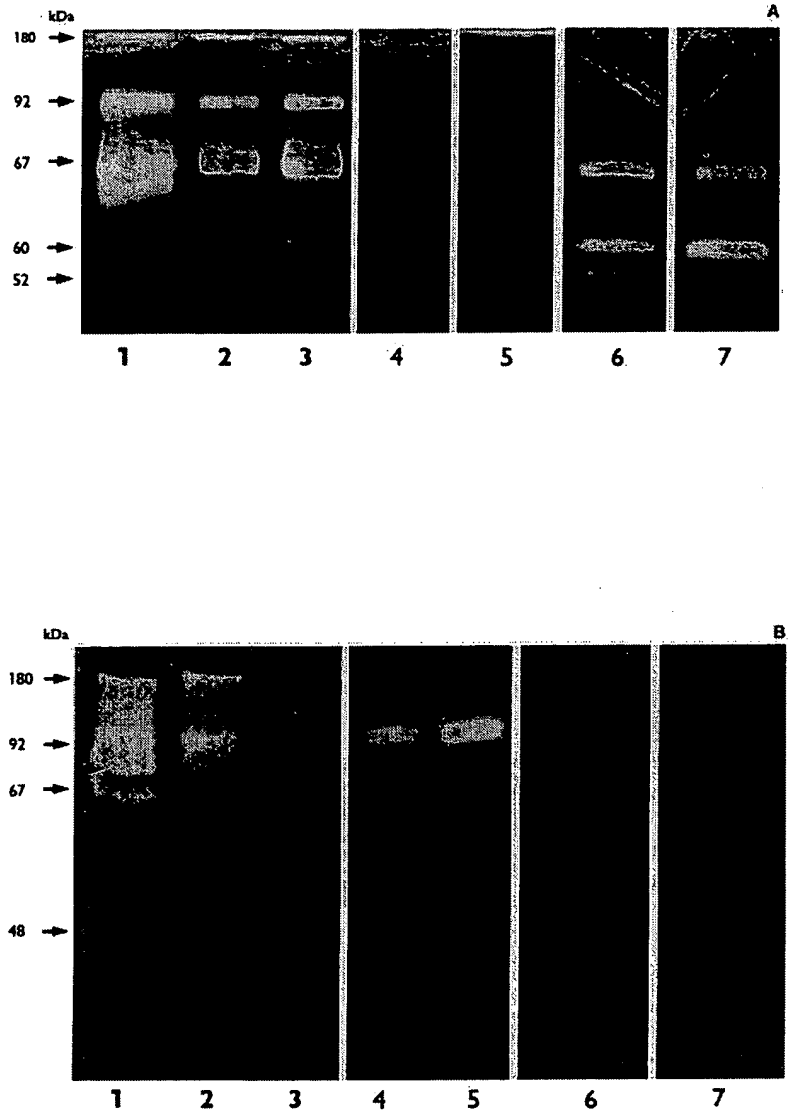
Strong evidence has been provided, suggesting that implantation of endometrial tissue is associated with matrix metalloproteinase (MMP) expression (17, 18). In the human reproductive cycle, expression of MMPs and their tissue inhibitors (TIMPs) is significant during the menstrual phase and is triggered by decreasing concentrations of luteal progesterone (14–16). Most studies on endometriosis and MMPs investigated the expression of MMPs and TIMPs in PF, endometriotic lesions, and cyclic endometrium in the presence of endometriosis. Although these studies invariably suggest a pivotal role of MMPs in the development of endometriosis, no direct evidence has been provided supporting this contention (23–26).

The present study shows that shed menstrual endometrium, the tissue from which endometriosis presumably originates, rather than mechanically obtained cyclic endometrium, expresses various MMPs and TIMPs several hours after shedding and continues to do so after 24 hours of culture. The cryostat sections showed expression of MMP-7 in epithelial cells and also some diffuse staining in the stroma. This can be explained by the fact that MMP-7 is an enzyme and after secretion it can diffuse in the interstitial space of stroma. This has also been observed by Rodgers and coworkers (27). The limited expression of MMP-7 in our cultures can be explained by the fact that the cultures predominantly consist of stromal cells. If epithelial cells were present in the cultures, these cells expressed MMP-7.

Matrix metalloproteinase-9 was expressed only weakly in cryostat sections and hardly in cultures, whereas zymography and Western blot analysis clearly showed the presence

FIGURE 3

(A), Gelatin zymography. (B), Casein zymography, menstrual serum cycle days 1, 2, and 3, (*lanes 1–3*), peritoneal fluid cycle days 3 and 26 (*lanes 4 and 5*), and conditioned medium (*lanes 6 and 7*).



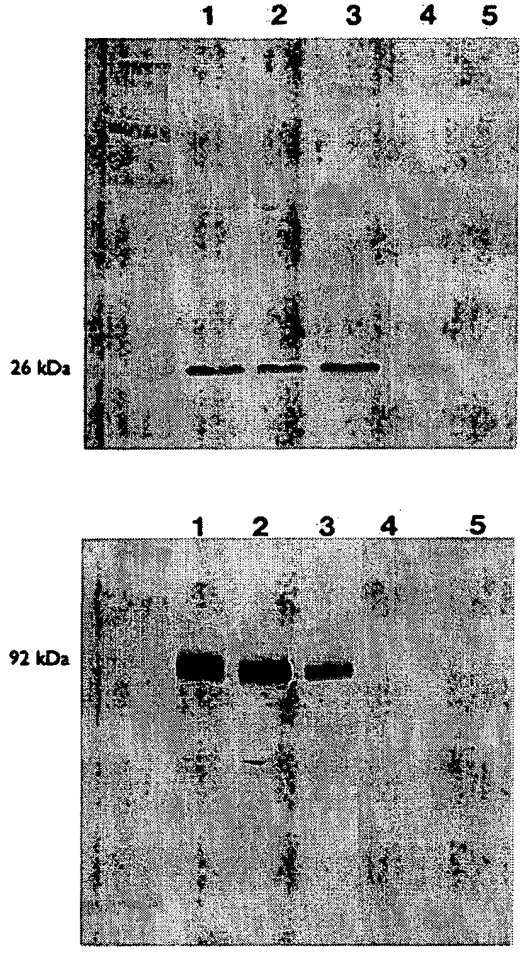
Koks. MMPs and TIMPs. Fertil Steril 2000.

of MMP-9 in menstrual serum of the first 3 days of the cycle. This may be due to the fact that MMP-9 expression is immunolocalized to polymorphic nuclear neutrophils in menstrual tissue (10). With Western blot analysis, only MMP-7 and MMP-9 were observed as strong bands at the expected molecular weight for menstrual serum. For the other MMPs and TIMPs, bands at the expected molecular weights and multiple other faint bands were observed. This might either be due to the specificity of the antibodies or, alternatively, the faint bands represent active, glycolysated, and covalent forms of the MMPs. The latter might be a valid explanation because the antibodies against MMP-7 and MMP-9 were directed only against the latent enzyme form, whereas the other antibodies were directed against both the latent and active forms of the enzyme.

We have postulated the peritoneal environment to be a defense barrier, preventing adhesion of endometrial tissue by degrading retrogradely shed endometrial tissue to single cells and reducing adhesion molecule expression (28). Proteases in the PF are thought to be responsible for this degradation and loss of adhesion molecule expression (28).

FIGURE 4

Western blots stained for (*top*) MMP-7 and (*bottom*) MMP-9, menstrual serum cycle days 1, 2, and 3 (*lanes 1–3*), peritoneal fluid cycle days 3 and 26 (lanes 4 and 5).



Koks. MMPs and TIMPs. Fertil Steril 2000.

However, with use of zymography and Western blot analysis, we have demonstrated that protease activity is significantly lower in PF than in menstrual effluent. The MMP activity in PF is, therefore, more likely the result of refluxed menstrual effluent rather than being produced by activated resident macrophages. Furthermore, after 24 hours of culture, menstrual endometrial tissue is still capable of expressing MMPs and TIMPs. This suggests that endometrial tissue on entering the abdominal cavity and after adhering to the (sub)peritoneal lining, can produce MMPs, which could facilitate invasion of these cells. Therefore, the amount of retrograde menstruation may be an important risk factor in the development of endometriosis.

In conclusion, the results of this study, together with results of earlier studies (17, 18), suggest that retrogradely shed menstrual endometrium, after adhering to the submesothelial layer, uses its own MMP activity to invade the extracellular matrix, resulting in the development of endometriotic lesions.

References

1. Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. Matrix metalloproteinases: a review. Crit Rev Biol Med 1993;4:197–250.
2. Hulboy DL, Rudolph LA, Matrisian LM. Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod 1997;3:27–45.
3. Huppertz B, Kertschanska S, Demir AY, Frank HG, Kaufman P. Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 1998;291:133–48.
4. Matrisian LM. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 1990;6:121–5.
5. Salamonsen LA, Woolley DE. Matrix metalloproteinases and their tissue inhibitors in endometrial remodelling and menstruation. Reprod Med Rev 1996;5:185–203.
6. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–73.
7. Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, et al. Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest 1994;94:946–53.
8. Matrisian LM, Gaire M, Rodgers WH, Osteen KG. Metalloproteinase expression and hormonal regulation during tissue remodeling in the cycling human endometrium. Contrib Nephrol 1994;107:94–100.
9. Kokorine I, Marbaix E, Henriet P, Okada Y, Donnez J, Eeckhout Y, et al. Focal cellular origin and regulation of interstitial collagenase (matrix metalloproteinase-1) are related to menstrual breakdown in the human endometrium. J Cell Sci 1996;109:2151–60.
10. Jeziorska M, Nagase H, Salamonsen LA, Woolley DE. Immunolocalization of the matrix metalloproteinases gelatinase B and stromelysin 1 in human endometrium throughout the menstrual cycle. J Reprod Fertil 1996;107:43–51.
11. Jeziorska M, Salamonsen LA, Woolley DE. Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. Biol Reprod 1995;53:312–20.
12. Busiek DF, Vijaykumar B, Nehring LC, Parks WC, Welgus HG. Matrilysin expression by human mononuclear phagocytes and its regulation by cytokines and hormones. J Immunol 1995;154:6484–91.
13. Shi W, Mognetti B, Campana A, Bischof P. Metalloproteinase secretion by endometrial leucocyte subsets. Am J Reprod Immunol 1995;34:299–310.
14. Marbaix E, Kokorine I, Henriet P, Donnez J, Courtoy PJ, Eeckhout Y. The expression of interstitial collagenase in human endometrium is controlled by progesterone and by oestradiol and is related to menstruation. Biochem J 1995;305:1027–31.
15. Osteen KG, Rodgers WH, Gaire M, Hargrove JT, Gorstein F, Matrisian LM. Stromal-epithelial interaction mediates steroidal regulation of metalloproteinase expression in human endometrium. Proc Natl Acad Sci USA 1994;91:10129–33.
16. Schatz F, Papp C, Toth-Pal E, Lockwood CJ. Ovarian steroid-modulated stromelysin-1 expression in human endometrial stromal and decidual cells. J Clin Endocrinol Metabol 1994;78:1467–71.
17. Spuijbroek MDEH, Dunselman GAJ, Menheere PPCA, Evers JLH. Early endometriosis invades the extracellular matrix. Fertil Steril 1992;58:929–33.
18. Bruner KL, Matrisian LM, Rodgers WH, Gorstein F, Osteen KG. Suppression of matrix metalloproteinases inhibits establishment of ectopic lesions by human endometrium in nude mice. J Clin Invest 1997;99:2851–7.
19. Koks CAM, Dunselman GAJ, de Goeij AFPM, Arends JW, Evers JLH. Evaluation of a menstrual cup to collect shed endometrium for in vitro studies. Fertil Steril 1997;55:560–4.
20. Koks CAM, Groothuis PG, Dunselman GAJ, de Goeij AFPM, Evers JLH. Adhesion of shed menstrual tissue in an in vitro model using amnion and peritoneum: a light and electron microscopic study. Hum Reprod 1999;14:816–22.
21. Mollenhauer HH, Morré DJ, Rowe LD. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochem Biophys Acta 1990;1031:225–46.
22. Fisher SJ, Cui T, Zhang L, Hartman L, Grahl K, Guo-Yang Z, et al. Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. J Cell Biol 1989;109:891–902.

23. Keisler LW, Keisler DH, McIntusch EW, Surewicz K, Nothnick WB, Sharpe-Timms KL. Tissue Inhibitor of metalloproteinase-1 (TIMP-1) concentrations are reduced in peritoneal fluid of women with pelvic endometriosis, pain, infertility. J Soc Gynecol Invest 1996;3(2 Suppl): 193a.
24. Kokorine I, Nisolle M, Donnez J, Eeckhout Y, Courtoy PJ, Marbaix E. Expression of interstitial collagenase (MMP-1) is related to the activity of human endometriotic lesions. Fertil Steril 1997;68:246–51.
25. Saito T, Mizumoto H, Kuroki K, Fujii M, Mori S, Kudo R. Expression of MMP-3 and TIMP-1 in endometriosis and the influence of danazol. Acta Obstet Gynaecol Jpn 1995;47:495–6.
26. Sharpe-Timms KL, Keisler LW, McIntush EW, Keisler DH. Tissue inhibitor of metalloproteinase-1 concentrations are attenuated in peritoneal fluid and sera of women with endometriosis and restored in sera by goadotrophin-releasing hormone agonist therapy. Fertil Steril 1998; 69:1128–34.
27. Rodgers WH, Osteen KG, Matrisian LM, Navre M, Giudice LC, Gorstein F. Expression and localisation of matrilysin, a matrix metalloproteinase, in human endometrium during the reproductive cycle. Am J Obstet Gynecol 1993;168:253–60.
28. Van der Linden PJQ, de Goeij AFPM, Dunselman GAJ, Erkens HWK, Evers JLH. Amniotic membrane as an in vitro model for endometrium-extracellular matrix interactions. Gynecol Obstet Invest 1998;57:7–11.

Exhibit J

1: Thromb Haemost. 2000 Nov;84(5):876-81.

Short-term effect of surgical trauma on rat peritoneal fibrinolytic activity and its role in adhesion formation.

Hellebrekers BW, Trimbos-Kemper GC, Bakkum EA, Trimbos JB, Declerck PJ, Kooistra T, Emeis JJ.

Department of Gynecology, Leiden University Medical Center, The Netherlands. bwjh@xs4all.nl

BACKGROUND: Fibrin deposition, the primary step in the formation of post-surgical adhesions, is the result of a disbalance between the fibrin-forming and the fibrin-dissolving capacity of the peritoneum. Literature data suggest a transient reduction in local plasminogen activator activity after peritoneal trauma, which results in a reduction of fibrinolysis and permits deposited fibrin to become organized into fibrous, permanent adhesions. In the present study, the fibrinolytic parameters tissue-type plasminogen activator (tPA; antigen and activity) and plasminogen activator inhibitor type-1 (PAI-1; antigen and activity) were measured in peritoneal fluid, in peritoneal biopsies and in plasma to establish the time course of changes in fibrinolytic activity. DESIGN: A standardized peritoneal adhesion model in the rat. OUTCOME MEASURES: Analysis, over a 72-h period following surgical trauma. of the main fibrinolytic parameters in peritoneal lavage, in biopsies of damaged and undamaged peritoneum, and in plasma, and determination of fibrin and fibrin(ogen)-degradation products in peritoneal lavage fluid. RESULTS: At all time intervals, tPA antigen was found to be about six-fold increased in peritoneal lavage after surgical trauma. This significant rise in tPA antigen was accompanied by a large increase in its main inhibitor PAI-1, resulting in tPA activity levels similar to, or slightly higher than, those found in control animals. tPA activity was lowest at 4 h and increased thereafter. Also in biopsies from damaged peritoneum, tPA antigen was significantly increased. Tissue tPA activity was also lowest at 4 h, after which it increased, significantly so at 24 and 72 h. Similar, though smaller, changes were seen in the biopsies from undamaged areas of the peritoneal wall in operated rats. PAI-1 (antigen and activity) was not detected in peritoneal biopsies. Fibrin-related material (especially fibrin monomer/fibrinogen, an indicator of forming fibrin)

in peritoneal fluid was slightly increased at 4 h, and abundantly present at 16 and 24 h, returning to control levels at 72 h. Fibrin degradation products were always present. From 2 h onward, adhesions were found. CONCLUSIONS: In contrast to the view that adhesions are formed as a result of a reduced fibrinolytic activity, our results demonstrate that tPA activity remained unchanged or slightly increased after surgical trauma, and point to increased fibrin formation rather than diminished fibrinolytic activity as the main cause of fibrin deposition after peritoneal trauma. Therapies directed at prevention of adhesion formation should therefore aim at avoiding massive fibrin production and at promoting fibrinolytic activity during the early period after trauma.

PMID: 11127871 [PubMed - indexed for MEDLINE]

Exhibit K

ELSEVIER

The American Journal of Surgery 181 (2001) 301–308

The American Journal of Surgery

Limits of peritoneal cytokine measurements during abdominal lavage treatment for intraabdominal sepsis

Stefan Scheingraber, M.D.[a,b,*], Frank Bauerfeind[b], Judith Böhme[b], Henning Dralle, M.D.[b]

[a]*Department of General Surgery, Rheinische Friedrich-Wilhelms-University, Sigmund Freud Strasse 25, D-53105 Bonn, Germany*
[b]*Department of General Surgery, Martin Luther University, Halle/Saale, Germany*

Manuscript received July 5, 2000; revised manuscript November 22, 2000

Abstract

Background: Monitoring of peritoneal cytokine concentrations of tumor necrosis factor (TNF)-α was recommended for early detection of severe postoperative complications. In the present study the clinical application of cytokine monitoring was examined in the treatment course of severe peritonitis.

Methods: Nineteen patients with secondary peritonitis were followed up during 75 abdominal lavages. Serum and peritoneal interleukin (IL)-6, IL-8, and IL-10 and TNF-α were measured before the surgical intervention, after 1 hour, 3 hours, 6 hours, and 24 hours. Additionally, cardiorespiratory parameters, osmolarity, C-reactive protein, and total leucocyte count were recorded.

Results: Serum and peritoneal cytokine concentrations did not correlate to each other as well as to the observed cardiorespiratory parameters. Peritoneal cytokine concentrations were 10- to 1000-fold higher to serum concentrations and showed an intermittent wash out. There were no differences in determined cytokine concentrations between survivors and nonsurvivors

Conclusions: Once elevated, peritoneal cytokine measurements offer no new diagnostic or prognostic tool in abdominal lavage peritonitis treatment.

Keywords: Intraabdominal sepsis; Peritoneal cytokine release; Cardiorespiratory function; Abdominal lavage therapy; Outcome

Cytokines are a group of proteins produced by a variety of cells playing an active role in the immune system. During the last decade many data have been evolved to determine the diagnostic role of serum cytokine concentrations found under several clinical conditions like after burn trauma, hemorrhage, soft tissue trauma, after elective surgery, in patients with pancreatitis, and in patients suffering from sepsis due to different origins. More fragmentary is our current view of the value of cytokine measurements in the fluid drained from the abdominal cavity. Recently, cytokine monitoring of peritoneal tumor necrosis factor (TNF)-α has been recommended in the early detection of severe intraabdominal complications after elective surgery [1]. In intraabdominal sepsis, where the peritoneal cavity is the inflammatory focus, the role of peritoneal cytokine peak concentrations or their elimination kinetic is still unclear. Whereas one study reported significant differences of TNF-α and elastase levels in the peritoneal exsudate after planned relaparotomy between survivors and nonsurvivors from intraabdominal sepsis [2], these results were not reproducible by another study showing no differences in peritoneal interleukin (IL)-8 and TNF-α concentrations in the perioperative course after extensive abdominal lavage between survivors and nonsurvivors [3]. Although it is now accepted that the host defense is a result of the balance of proinflammatory as well as antiinflammatory factors [4], there are no clinical data of peritoneal antiinflammatory cytokine measurements in secondary peritonitis, eg, IL-10, so far. Moreover, there are only a few data describing the cardiorespiratory function of the patients with intraabdominal sepsis in the course of the abdominal lavage procedure [5]. We did this study to clarify the role of peritoneal proinflammatory and antiinflammatory cytokines in intraabdominal sepsis, the kinetics of systemic and peritoneal compartimentalized cytokine concentrations in the course of abdominal lavage, and their possible relationship to cardiorespiratory function and to outcome.

* Corresponding author. Tel.: +49-228-287-5109; fax: +49-228-287-4317.
E-mail address: scheingraber@chir.uni-bonn.de.

0002-9610/01/$ – see front matter
PII: S0002-9610(01)00587-6

Patients and methods

Patients and treatment plan

From November 1998 to December 1999 19 patients with secondary peritonitis admitted to the Department of General Surgery of the University Hospital were observed during 75 abdominal lavage procedures performed in the operating theater or directly on the intensive care unit. Our treatment strategy for diffuse peritonitis is to leave the abdomen open and include the patients in a lavage program with 24-hour or longer intervals depending on the judgment of the attending surgeon. For the lavage, normal saline without any antimicrobial ingredients was used. During the lavage program, patients were treated with mechanical ventilation, analgosedation, fluid resuscitation with respect to the central venous pressure monitoring, and vasopressor therapy or inotropic drugs administered to maintain adequate invasive monitored peripheral arterial pressures.

Sampling

Arterial blood samples and samples from a drain, which was inserted into the pouch of Douglas, were taken before lavage (t0), after 1 hour (t1), after 3 hours (t2), 6 hours (t3), and 24 hours (t4) in endotoxin-free tubes. In most cases the drain fluid was suctioned with a syringe from the drain system and not taken from the collection bag. The samples were immediately kept cool, centrifuged and frozen within 30 minutes at 70°C until processing. The Institutional Review Board waived the need for informed consent as all measurements were done from the patients serum taken for clinical routine reasons and drain fluid, which was usually wasted.

Assays for TNF-α, IL-6, IL-8, and IL-10, C-reactive protein, and method of leucocyte count

TNF-α, IL-6 and IL-8 were measured with the fast immunoluminescence method using the Immulite (DPC, Bad Nauheim, Germany). The detection limits were 1.7 pg/mL for TNF-α, 5.0 pg/mL for IL-6, and 2.0 pg/mL for IL-8. For IL-10 an ELISA (Milenia; DPC, Bad Nauheim, Germany) was used according to the manufacturer's instructions. The detection limit for this test was 3.0 pg/mL. All measurements were done in duplicate. For intraperitoneal cytokine determinations the samples were diluted up to 1:300000 with dilution solution provided by the manufacturer. C-reactive protein (Synchron Cx-System, Beckman Coulter Comp.) and white blood cell count ([WBC] NE Alpha K 4500, Sysmex Comp.) were measured automatically with a specific C-reactive protein antibody and an electrical resistance system in the hospital laboratory and recorded from the patients charts.

Calculation of cardiocirculatory and respiratory parameters

The mean arterial pressure (MAP) was taken from the monitoring of the radial artery catheter. As septic shock may be mimicked by apparent sufficient mean arterial pressure due to administration of vasopressors, eg, norepinephrine, epinephrine, the ratio of mean arterial pressure/vasopressor level, calculated as multiples of 0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (MAP/VP) was suggested by Sautner [5]. But for doses of 0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$ this ratio would not be different compared with the mean arterial pressure and other drugs, eg, inotropic substances like doputamine and dopamine might also increase the mean arterial pressure. Moreover, the administered volume of crystalloid or colloidal infusion solutions, as well as administered units of fresh frozen plasma and packed red blood cells also influenced hemodynamic stability. We therefore formed the mean arterial pressure/drug and fluid ratio (MAP/DF) following these criteria: MAP/DF = MAP/(norepinephrine dose in multiplies of 0.01 μg/kg) + (epinephrine dose in multiplies of 0.01 μg/kg) + (dopamine dose in multiplies of 0.01 μ/kg) + (dopexamine dose in multiplies of 0.01 μ/kg) + (total volume of administered crystalloid solutions in mL/3,000) + (total volume of administered colloidal solutions in mL/1,000) + (number of administered units of fresh frozen serum/4) + (number of administered packed red blood cells/2). The quotients for crystalloid solutions, colloidal solutions, fresh frozen serum, and packed red blood cells were chosen based on our personal observation in the ability to maintain hemodynamic stability in surgical ICU patients. For the description of the pulmonary oxygenation, the Horowitz quotient, as referred to in the German literature, was calculated as the ratio of pO_2/oxygen fraction in the inspiratory air (FiO_2) [6].

Statistical analysis

Data are presented with boxplots in the Figs. on the left side. As there was a large variability and a wide range of many data, the mean values with their ± SEM of the changes (Δ-values) from one to the next time point were shown in the Figs. on the right side, first, for the group of survivors and second, for the group of nonsurvivors. On the left side in Figs. 2 and 3 first the cytokine serum concentrations and than the cytokine peritoneal concentrations are shown. Statistical analyses were carried out with the SPSS package (SPSS, Inc. SPSS for Windows, Chicago, Illinois). Boxplots and Δ-values of the different time points were compared with the baseline levels at t0 using the *t* test. For subgroup analyzes two-way repeated analysis of variance (ANOVA) as well as the *t* test for intragroup comparisons were performed. Probabilities <0.05 were considered as statistically significant.

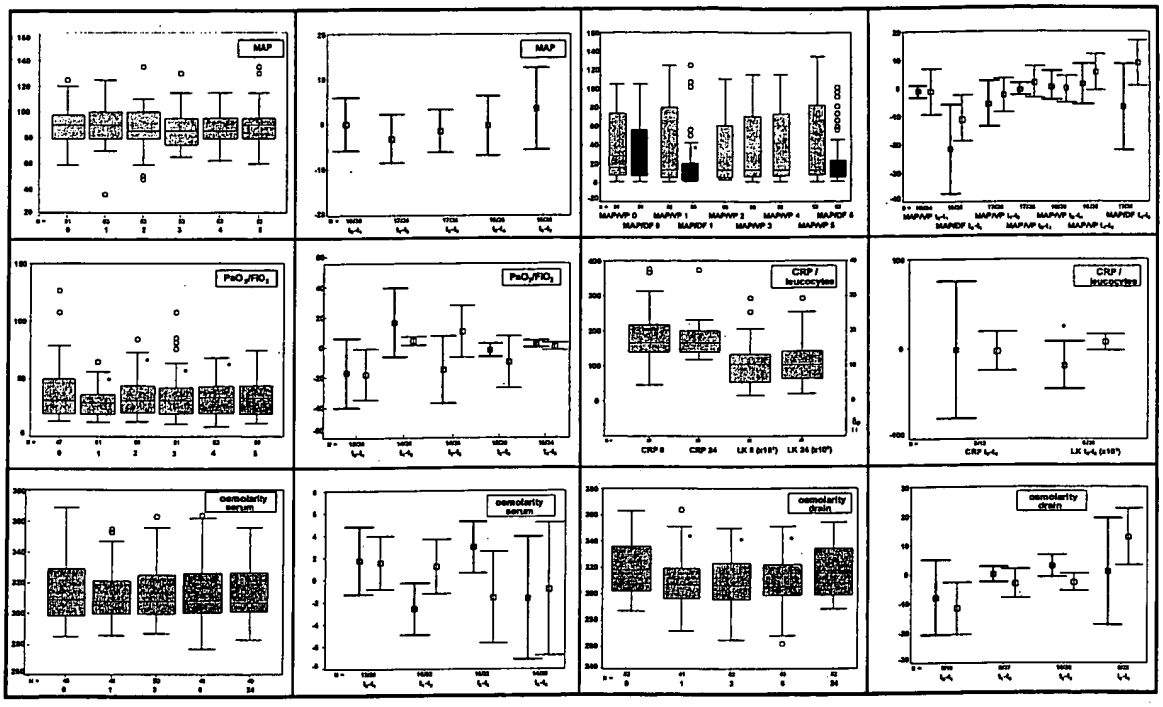


Fig. 1. Cardiorespiratory and conventional inflammatory parameters.

Results

Patients

Twelve male and 7 female patients underwent a total number of 116 (mean 6.1, range 1 to 32) abdominal lavages. Seventy-five lavages (65%) could be followed up in this study. Seventy percent of the measured lavages were performed in the operating theater and 30% directly on the bedside, to avoid any transport mishaps for critical patients. The hospital mortality among the study group of 19 patients was 47%. Mean hospital stay was 35.9 days (range 5 to 86). The abdomen has been left open in mean for 11 days (range 1 to 61). The origin of the peritonitis were spontaneous sigmoid perforation (n = 5), postoperative duodenal and small bowel leakage (n = 4), gastric/duodenal ulcer perforation (n = 2), postoperative necrotizing pancreatitis (n = 2), acute necrotizing pancreatitis (n = 2), perforated appendicitis (n = 1), gangrenous cholecystitis (n = 1), anastomotic failure (n = 1), and iatrogenic duodenal perforation (n = 1).

Cardiorespiratory function

Fig. 1 illustrates cardiorespiratory parameters during the different time points in the course of abdominal lavage of the whole study group. There was a marked range in the MAP, the MAP/VP, the MAP/DF, and the respiratory index. Neither the MAP nor the MAP/VP showed significant changes in the course of the procedure taken all patients together or regarding only the changes between two time points in the group of the survivors and the nonsurvivors. In contrast, there was a significant decrease of MAP/DF from the begin of the abdominal lavage to the first 15 minutes, which was reversible 24 hours after the procedure. Again, there was no significant difference in the amount of this decrease between survivors and nonsurvivors. After abdominal lavage there was a significant decrease in the pO_2/FiO_2 from 46 torr at baseline level to 29 torr at 1 hour, which continued to increase and returned to the baseline level not before 24 hours. The amount of the changes of the pO_2/FiO_2 were not different between survivors and nonsurvivors.

Conventional inflammatory parameters and osmolarity

In Fig. 1 also the changes in the C-reactive protein and WBC are demonstrated. There were no differences in these two parameters the day after the abdominal lavage compared with the baseline levels before this procedure in the whole study group. There was also great variability in the changes of C-reactive protein and WBC. In this context, survivors showed a significant larger variability in WBC than nonsurvivors. As illustrated, serum osmolarity showed a broad range in the patients but the changes for the individual patient remained only small and without differences between survivors and nonsurvivors. More marked changes were seen in the peritoneal osmolarity measured from the drain fluid. The lavage led to a significant decrease from 321 mosm/L at baseline level to about 310 mosm/L at 1 hour, 3

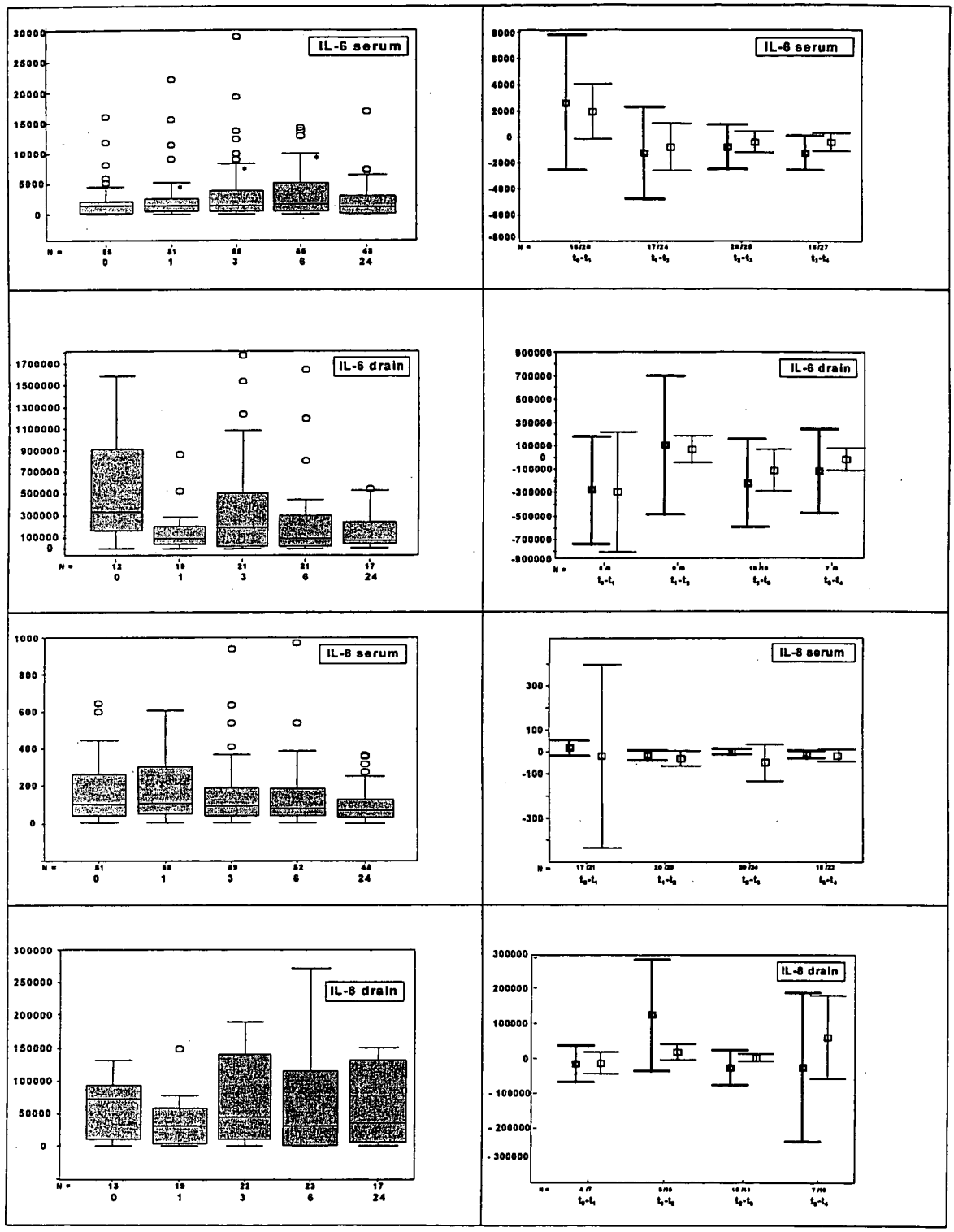


Fig. 2. Serum and peritoneal interleukin-6 and interleukin-8 kinetics.

hours, and 6 hours. One day after the abdominal lavage, the osmolarity reached the baseline level again. There were no differences in the intensity of these changes between time points in the group of the survivors and the nonsurvivors.

Serum and peritoneal IL-6, IL-8, TNF-α and IL-10

As shown in Fig. 2, the range as well as the mean values of serum IL-6 values increased significantly in the course of

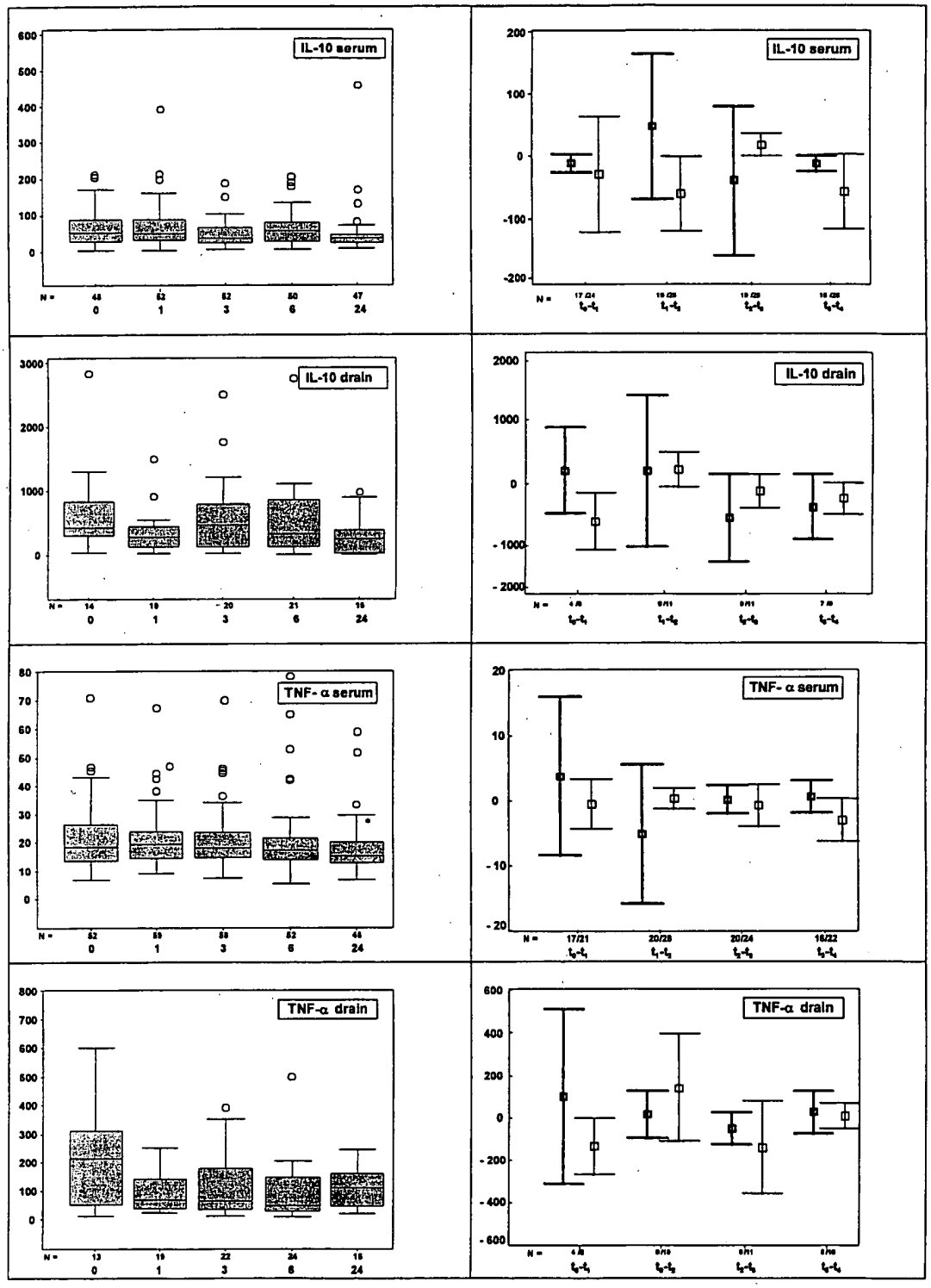


Fig. 3. Serum and peritoneal interleukin-10 and tumor necrosis factor-α kinetics.

6 hours after the abdominal lavage from 2,135 pg/mL to 3,724 pg/mL at 1 hour, 3,524 pg/mL at 3 hours, and 3,214 pg/mL at 6 hours, and returned to baseline levels of about 2,342 pg/mL 24 hours postoperatively. Additionally, there were many extremely high individual IL-6 values up to 30,000 pg/mL during the time points. Peritoneal IL-6 concentrations were 100- to 1,000-fold higher compared with the values measured in the serum. There was only a trend of a decrease of peritoneal IL-6 from baseline level to 1 hour, which lacked statistical significance ($P = 0.12$). Twenty-four hours after the abdominal lavage, the range of peritoneal IL-6 values was much smaller compared with the

values at baseline level. The analysis of the Δ-values showed also a broad range in the serum as well as peritoneal IL-6 concentrations during the different time points, but there was no different behavior between survivors and nonsurvivors.

Serum and peritoneal IL-8 concentrations showed no significant changes from baseline level to 24 hours postoperatively. There were also some individual extremely high IL-8 serum levels up to 1,000 pg/mL measured during the study. Peritoneal IL-8 concentrations were about 300-fold higher than serum concentrations. Individual changes showed a broad range of serum IL-8 from baseline level to 1 hour in the nonsurvivors, but this was not significantly different ($P = 0.87$) from the survivors. In the later course of abdominal lavage there were only small individual changes in serum IL-8 concentrations in survivors and nonsurvivors and relatively large but not significantly different individual changes of peritoneal IL-8 concentrations in survivors compared with nonsurvivors.

In the course of abdominal lavage there was a slight but significant decrease of serum TNF-α from 21 to 18 pg/mL the first 24 hours after abdominal lavage. There were some individual extreme values up to 80 pg/mL found in the serum. Peritoneal TNF-α concentrations were about 10-fold higher than serum concentrations and showed no significant change during the time points. Changes in the serum and peritoneal TNF-α concentrations during the different time points were in the similar range in survivors and nonsurvivors.

Serum and peritoneal IL-10 concentrations showed no changes in the course of abdominal lavage (Fig. 3). Peritoneal concentrations were up to 10-fold higher than serum concentrations with peak concentrations of about 2,800 pg/mL. Changes in individual cases showed some remarkable ranges which, however, were not different between survivors and nonsurvivors.

Subgroup analysis for differences in cardiorespiratory function and serum and peritoneal cytokine concentrations between survivors and nonsurvivors

For further analysis patients were divided in two subgroups according the criteria for the extent of the surgical trauma and the order of the measured lavage in the context of the treatment course of the abdominal lavage given in Table 1. There were 7 cases with primary laparotomy (group 1), 19 cases with secondary laparotomy and minor surgical procedures (group 2), and 38 cases with lavage only (group 3). Between group 1, group 2, and group 3 ANOVA revealed significant differences in $\Delta_{15\ min-24\ h}$ MAP/DF (group 1 and 2 $P = 0.045$, group 1 and 3 $P = 0.046$), in $\Delta_{6-24\ h}$ serum IL-6 (group 1 and 2 $P = 0.035$), in $\Delta_{3-6\ h}$ serum IL-10 (group 1 and 2 $P = 0.012$, group 1 and 3 $P = 0.012$), and in $\Delta_{3-6\ h}$ serum TNF-α (group 1 and 3 $P = 0.029$). Only the subgroup of patients with lavage only (group 3) could be further analyzed regarding a difference between survivors (n = 14) and nonsurvivors (n = 24). Nonsurvivors in this subgroup had significant higher pO_2/FiO_2 ratios after 1 hour ($P = 0.049$), after 6 hours ($P = 0.001$), and after 24 hours ($P = 0.008$). Additionally, serum osmolarity was significant higher in the nonsurvivors 1 hour ($P = 0.038$), 3 hours ($P = 0.017$), 6 hours ($P = 0.053$), and 24 hours ($P = 0.028$) after the abdominal lavage procedure. Nonsurvivors showed higher serum IL-8 after 1 hour, higher serum TNF-α after 24 hours, and lower peritoneal TNF-α concentrations at the start of the surgical intervention compared with the survivors.

Furthermore, in 8 cases first laparotomy for suspected peritonitis was performed without definitive abdominal closure, and in 3 cases a single laparotomy with definitive abdominal closure, also named standard procedure, was done. In 9 cases the last performed abdominal lavage was

Table 1
Subgroups according to the extent of surgical trauma and order in the treatment course of lavages

	Procedure	Number (percentage) measured lavages
Classification of trauma		
I	Laparotomy (with/without abdominal closure) Visceral resection (eg, Hartmann procedure, colonic segmental resection, gastric/duodenal ulcer excision)	7 (10.9%) DOD = 5 A&W = 2
II	Suturing of thinned bowel wall Extensive necrectomy Time-spending bowel preparation Difficult abdominal closure (eg, subcutaneous mobilization)	19 (29.7%) DOD = 14 A&W = 5
III	Lavage only Tension-free abdominal wall closure	38 (59.4%) DOD = 24 A&W = 14
Order of lavages		
I	First laparotomy for clinical suspected peritonitis (also laparotomy *after* elective surgery with suspected postoperative peritonitis, eg, anastomotic failure) without definitive abdominal closure, because of planned revision the next 24–48 hours	8 (12.5%) DOD = 5 A&W = 3
II	Last performed abdominal lavage: procedure is completed with definitive abdominal closure or the peritoneal cavity is no longer accessible because of granulation tissues or patient died before abdominal closure	9 (14.1%) DOD = 5 A&W = 4
III	Classic standard procedure with one laparotomy including definitive abdominal closure	3 (4.7%) DOD = 2 A&W = 1
IV	Abdominal lavages during abdomen apertum (between groups I and II)	44 (68.7%) DOD = 31 A&W = 13

DOD = dead of disease; A&W = alive and well.

measured. In the most cases, samples were taken during planned abdominal lavages with open abdomen, and only this subgroup of 44 patients was further analyzed. Once again, the pO_2/FiO_2 was significantly higher in the group of nonsurvivors 6 hours ($P = 0.001$) and 24 hours ($P = 0.009$) after the abdominal lavage. Additionally, in the nonsurvivors the serum osmolarity was significantly higher 3 hours ($P = 0.019$) and 24 hours ($P = 0.013$) after the procedure, and also the peritoneal osmolarity was higher ($P = 0.007$) before the operation. There were only changes in the cytokines between survivors and nonsurvivors, for serum IL-10 with higher values in the nonsurvivors before the operation ($P = 0.007$), for serum TNF-α with lower values in the nonsurvivors 24 hours after the operation ($P = 0.02$), and still significantly lower leucocytes in the nonsurvivors before the operation (0.049).

Comments

It is well known from experimental studies that peritoneal mesothelial cells [7,8] as well as peritoneal macrophages [9,10] can be regarded as a profound source of different intraabdominal cytokine production. Clinical studies in spontaneous peritonitis in ambulatory peritoneal dialysis patients and in liver cirrhosis patients with infected ascites were the first reports of high levels of IL-6, IL-10, and TNF-α in the peritoneal fluid [11–13]. Cytokines have also been detected in the peritoneal exudate from patients after abdominal surgery and were correlated to the bacterial contamination, the blood loss and the duration of surgery [14]. Moreover, much higher peritoneal cytokine concentrations in the peritoneal fluid drained after elective surgery were supposed to be the origin of much lower detectable serum levels [15]. This hypothesis was further supported by an anecdotal report of IL-6 and IL-8 positive mRNA leucocytes in the postoperative thoracic and abdominal fluid, but not in the peripheral blood [16]. Actually, it became not clear whether there is a spillover effect of cytokines from the compartmentalized peritoneal cavity to the intravascular compartment.

Another important question for the efficient treatment of peritonitis is the still high mortality of about 12% even after antibiotic and operative therapy resulted in a sterile peritoneal cavity [17]. One possible explanation for this phenomenon might be that the recurrent operative trauma in the planned abdominal lavage treatment acts as a second hit for already primed immune cells, escalating the systemic inflammatory response syndrome.

The contradicting studies in patients with laparotomy for peritonitis done by Holzheimer et al [2] and Fröhlich et al [3] had both, essentially, no follow-up of repeated abdominal lavages. In this study we examined the treatment course of the patients, which includes that successive single lavage procedures were followed up for 24 hours and cytokine measurements were done simultaneously in the peritoneal fluid and in the serum. With this study design we found tremendously high peritoneal values for all measured cytokines and a broad range in all measured parameters, making statistical analysis more difficult. As the cytokines were sampled via a drain that was placed in the pouch of Douglas, it is possible that we measured only the areas immediately about the drain tract itself, which might have been not representative for the whole abdominal cavity. Nevertheless, it was our treatment plan of the abdominal lavage to mobilize adherent intestinal loops as noninvasively as possible, to clean abscess formations between the intestinal loops, and to drain the lavage fluid running to the pouch of Douglas. A much smaller amout of fluid was drained directly via the laparostoma. To avoid errors due to collection in the drain bag, the peritoneal samples were aspirated directly from the drain tube using a syringe whenever possible. Additonally, owing to high peritoneal values, the centrifuged supernatants of the drainage fluid must have become diluted, which bears the risk for systematic dilution errors. Serum and peritoneal cytokines were always measured apart from the locus of their synthesis and biological action and were therefore compared with the "tip of a iceberg" [18]. In contrast to the spillover hypothesis, a better explanation of the cytokine kinetics in this study is the theory that the surgical trauma induced the intermittent increase of serum IL-6 and that the peritoneal IL-6 concentrations showed an intermittent decrease due to the washout and the dilution of lavage fluid. The suggestion of a dilutional effect is also supported by the decrease in peritoneal osmolarity. These findings may be therefore interpreted in terms of a compartmentalized and independent response of serum and peritoneal localized cytokines to the abdominal lavage.

In the perioperative course of the first laparotomy for clinically suspected peritonitis an association of circulatory and respiratory failure and increased concentration of cytokines like TNF-α or IL-6 were described [19]. To put some light on the cardiorespiratory parameters, in the treatment course of abdominal lavage the MAP/DF quotient, although a very rough simplification, was calculated. The MAP/DF quotient pays attention to the fact that the hemodynamic variables have always to be interpreted in the context of administered fluids and vasoactive or positive inotropic drugs. There were no clinically relevant respiratory and cardiocirculatory deteriorations during the perioperative period of abdominal lavage. The significant decrease in MAP/DF can illustrate that the administered fluid regime during surgery is sufficient to keep the hemodynamic parameters stable. The decrease in the paO_2/FiO_2 ratio should be interpreted with caution as the values before surgery and 1 hour after surgery may be influenced by an accidental high oxygen fraction used in the transport ventilation devices.

Finally, serum and peritoneal cytokine concentrations were examined for any use in outcome prediction, which was postulated first in the study done by Holzheimer et al [2]. In the study reported herein, a larger group of patients

with more abdominal interventions was included. More importantly, we formed subgroups in terms of the surgical trauma and the order of the abdominal lavages. The results of our study may be interpreted in the way that there is a overlap in the composition of both subgroups, as, for example, the first laparotomy is usually accompanied by a major surgical trauma than any single lavage. Essentially, the serum cytokine kinetics in this group of patients with their first laparotomy show the same pattern as in any elective abdominal surgery [20,21]. As expected, the group of patients with lavage only and lavage during abdomen apertum had the largest numbers of measured lavages, and some patients may be included in these groups several times, which might bias the results like the differences in the paO_2/FiO_2 between survivors and nonsurvivors. However, despite differences in osmolarity, which can be explained in context of the chronic hyperosmolar state of a surgical intensive care patient, there were no significant differences found in cytokine kinetics between survivors and nonsurvivors.

In conclusion, despite their pharmacokinetical properties compared with conventional parameters, eg, C-reactive protein [22], cytokines offer no new diagnostic tool in the treatment decision of patients suffering from severe diffuse peritonitis, although they can easily collected from the drain bag. These findings are in contrast to postoperative peritoneal cytokine monitoring for intraabdominal complications in elective surgery. Thus, once elevated, cytokine concentrations underlie many unspecific influencing factors. Like serum cytokines determinations, peritoneal cytokine measurements appear to detect only the tip of a iceberg, which—due to the tremendous amounts of cytokine levels—might be much higher. Therefore, future research should be focused on the local as well as transmigrated peritoneal cells themselves, which are producing these amounts of proinflammatory and antiinflammatory cytokines.

References

[1] van Berge Henegouwen MI, van der Poll T, van Deventer SJH. Peritoneal cytokine release after elective gastrointestinal surgery and postoperative complications. Am J Surg 1998;175:311–16.

[2] Holzheimer RG, Schein M, Wittmann DH. Inflammatory response in peritoneal exudate and serum of patients undergoing planned relaparotomy for severe secondary peritonitis. Arch Surg 1995;130:1314–20.

[3] Fröhlich D, Eiber RM, Jochum M, Billing A. Perioperative pattern of peritoneal interleukin 8, tumor necrosis factor-α, and granulocyte elastase release in human secondary peritonitis. Cytokine 1997;288–92.

[4] Bone RC. Sir Isaac Newton, sepsis, SIRS and CARS. Crit Care Med 1996;24:1125–8.

[5] Sautner T, Götzinger P, Redl-Wenzl EM, et al. Does reoperation for abdominal sepsis enhance the inflammatory host response? Arch Surg 1997;132:250–5.

[6] Bone RC, Maunder R, Slounan G, et al. An early test of survival in patients with the adult respiratory distress syndrome. The PaO_2/FiO_2 ratio and its differential response to conventional therapy. Chest 1989;96:849–51.

[7] Lanfrancone L, Boraschi D, Ghiara P, et al. Human peritoneal mesothelial cells produce many cytokines (granulocyte-stimulating factor [CSF], granulocyte-monocyte-CSF, macrophage-CSF, interleukin-1 [IL-1] and IL-6) and are activated and stimulated to grow by IL-1. Blood 1992;80:2835–42.

[8] Betjes MGH, Tuk CW, Struijk DG, et al. Interleukin-8 production by human peritoneal mesothelial cells in response to tumor necrosis factor-α, interleukin-1, and medium conditioned by macrophages cocultured with Staphylococcus epidermidis. J Infect Dis 1993;168: 1202–10.

[9] McMasters KM, Cheadle WG. Regulation of macrophage TNFα, IL-1β, and Ia (1-Aα) mRNA expression during peritonitis is site dependent. J Surg Res 1993;54:423–30.

[10] Hadjiminas DJ, McMasers KM, Peyton JC, Cheadle WG. Tissue tumor necrosis factor mRNA expression following cecal ligation and puncture or intraperitoneal injection of endotoxin. J Surg Res 1994; 56:549–55.

[11] Propst T, Propst A, Herold M, et al. Spontaneous bacterial peritonitis is associated with high levels of interleukin-6 and its secondary mediators in ascitic fluid. Eur J Clin Invest 1993;23:832–6.

[12] Zemel D, Koomen GCM, Hart AAM, et al. Relationship of TNF-α, interleukin-6, and prostraglandins to peritoneal permeabilitiy for macromolecules during longitudinal follow-up of peritonitis in continous ambulatory peritoneal dialysis. J Lab Clin Med 1993;122:686–96.

[13] Lu Y, Hylander B, Brauner A. Interleukin-10, interferon gamma, interleukin-2, and soluble interleukin-2 receptor alpha detected during peritonitis in the dialysate and serum of patients on continous ambulatory peritoneal dialysis. Peritoneal Dial Int 1996;16:607–12.

[14] Tsukada K, Katoh H, Shiojima M, et al. Concentrations of cytokines in peritoneal fluid after abdominal surgery. Eur J Surg 1993;159: 475–9.

[15] Badia JM, Whawell SA, Scott-Coombes DM, et al. Peritoneal and systemic cytokine response to laparotomy. Br J Surg 1996:347–8.

[16] Cavaillon JM, Munoz C, Fitting C, et al. Circulating cytokines: the tip of the iceberg? Circ Shock 1992;38:145–52.

[17] Sakamoto K, Arakawa H, Mita S, et al. Elevation of circulating interleukin 6 after surgery: factors influencing the serum level. Cytokine 1994;6:181–6.

[18] Schein M, Wittmann DH, Holzheimer R, Condon RE. Hypothesis: compartmentalization of cytokines in intraabdominal infection. Surgery 1996;119:694–700.

[19] Függer R, Zadrobilek E, Götzinger P, et al. Perioperative TNFα and IL-6 concentrations correlate with septic state, organ function, and APACHE II scores in intra-abdominal infection. Eur J Surg 1995; 159:525–9.

[20] Baigrie RJ, Lamont PM, Kwiatkowski D, et al. Systemic cytokine response after major surgery. Br J Surg 1992;79:757–60.

[21] Kato M, Honda I, Suzuki H, et al. Interleukin-10 production during and after upper abdominal surgery. J Clin Anesth 1998;10:184–8.

[22] Ohzato H, Yoshizaki K, Nishimoto N, et al. Interleukin-6 as a new indicator of inflammatory status: detection of serum levels interleukin-6 and C-reactive protein after surgery. Surgery 1992;111:201–9.

Exhibit L

Kidney International, Vol. 63 (2003), pp. 298–305

Glucose degradation products in PD fluids: Do they disappear from the peritoneal cavity and enter the systemic circulation?

MARTIN ZEIER, VEDAT SCHWENGER, REINHOLD DEPPISCH, ULRIKE HAUG, KAI WEIGEL, U. BAHNER, CHRISTOPH WANNER, H. SCHNEIDER, THOMAS HENLE, and EBERHARD RITZ

Department of Internal Medicine, University of Heidelberg, Heidelberg; Gambro Corporate Research, Hechingen; Technical University of Dresden, Institute of Food Chemistry, Dresden; Nephrology Department, University of Würzburg, Würzburg; and Nephrology Center, Stuttgart, Germany

Glucose degradation products in PD fluids: Do they disappear from the peritoneal cavity and enter the systemic circulation?

Background. Glucose degradation products (GDP) are generated in peritoneal dialysis (PD) fluid during heat sterilization and storage. They are thought to adversely affect the peritoneal membrane. The fate of GDP within the peritoneal cavity has not been well characterized.

Methods. A clinical study was designed to determine (*1*) whether during the dwell in the peritoneal cavity GDP concentration decreases in the PD fluid as assessed by ex vivo formation of AGE; (*2*) whether exposure to GDP-containing PD fluids increases plasma fluorescence (as an index of plasma AGE concentration) as well as plasma carboxymethyllysine (CML) concentration; and (*3*) whether exposure to GDP-containing PD fluids adversely affects glycoprotein CA 125 concentration. A two-group crossover design was adopted comprising two consecutive observation periods of eight weeks each. Stable PD patients were exposed in random order either to conventional PD fluid (heat sterilized at pH 5.5) and subsequently to PD test fluid (or the 2 fluids in reverse order). The PD test fluid was sterilized using a multicompartment bag system separating highly concentrated glucose at pH 3 from the buffer solution. Conventional and test fluids differed with respect to concentrations of GDP, that is, 3-deoxyglucosone (118 vs. 12.3 μmol/L), methylglyoxal (5.3 μmol/L vs. below detection threshold), 3, 4-dideoxyglucosone-3-ene (10 μmol/L vs. below detection threshold) and acetaldehyde (226 vs. <1 μmol/L).

Results. The following results were obtained. First, methylglyoxal disappeared completely as early as two hours after intraperitoneal instillation of conventional PD fluid. Second, when spent conventional dialysate was recovered after a two hour and particularly an eight hour dwell and subsequently incubated ex vivo with 40 mg of human serum albumin, there was a continuous decrease of AGE-forming capacity, that is, less generation of fluorescence (AGE) and pyrraline (non-fluorescent Amadori product), and an increase of advanced oxidation protein products (AOPP) in the spent dialysate. Third, plasma fluorescence (exc. 350/em. 430 nm) as an index of circulating AGE compounds as well as plasma CML concentrations were significantly higher in the conventional PD fluid period versus low GDP PD fluid period. Fourth, CA 125 concentrations in spent dialysate were higher during the low GDP PD fluid period compared to the conventional PD fluid period.

Conclusion. Conventional PD fluid undergoes modifications during intraperitoneal dwell with a loss of AGE forming capacity, suggesting breakdown, precipitation or resorption of GDP in vivo. This is accompanied by an increase in plasma AGE compounds.

Key words: peritoneal dialysis, glucose degradation products, carbonyl compounds, oxidative stress, advanced glycation end products.

Received for publication March 19, 2002
and in revised form July 31, 2002
Accepted for publication August 7, 2002

Glucose as a highly reactive substance is easily degraded into glucose degradation products (GDP), which are toxic [1, 2] and can give rise to early or advanced glycation end products (AGE) [3, 4]. The formation of GDP is dependent on pH, temperature and time. In peritoneal dialysis (PD) fluids GDP are generated when glucose is heated under acidic conditions [5] as well as subsequently during storage of the sterilized fluids. The GDP comprise formaldehyde, glyoxal, methylglyoxal, 3-deoxyglucosone, 3,4-dideoxyglucosone-3-ene, and many substances that have not yet been identified chemically [6].

The reactions that GDP (i.e., carbonyl compounds) undergo in the peritoneal cavity and the benefit potentially derived from elimination of GDP are of great clinical interest. Initial studies [7, 8] show higher glycoprotein CA 125 concentrations as an index of mesothelial cell number and/or viability [9, 10] when low GDP PD fluids are used.

It was the purpose of the present prospective randomized, crossover study to confirm and extend these results by addressing two issues:

1. Do GDP disappear from the PD fluid during the dwell? This was assessed first by measuring methylglyoxal as a representative GDP compound and second by measuring ex vivo formation of AGE compounds and advanced oxidation protein prod-

ucts (AOPP) in spent PD dialysates as an index of GDP dependent glycation and oxidation capacity.
2. Do GDP escape from the peritoneal cavity and enter the systemic circulation? Plasma fluorescence, total and molecular weight-specific, as well as carboxymethyllysine (CML) concentrations were compared during the treatment periods using conventional and low GDP PD fluids, respectively.

METHODS

Study design

The study was performed as a multicentric, prospective, randomized, two-group crossover clinical trial comprising two consecutive observation periods of eight weeks each.

In random order patients were either first exposed to PD fluid with minimal concentrations of glucose degradation products (low GDP fluid, Gambrosol trio®; Gambro Co., www.gambro.de) during eight weeks and subsequently switched to a conventional PD fluid (high GDP fluid, Gambrosol®, Gambro Co.) for the subsequent eight weeks (group A). Alternatively, patients were started with high GDP fluid and were subsequently switched to low GDP fluid (group B). The 1.5% glucose-containing PD fluids differed with respect to pH (pH ~5.5 in high GDP vs. ~6.5 in low GDP fluid) and concentrations of GDP: methylglyoxal (5.3 vs. below detection limit of 2.8 μmol/L), and acetaldehyde (226 vs. below 1 μmol/L), 3-deoxyglucosone (118 vs. 12.3 μmol/L), 3,4-dideoxyglucosone-3-ene (10 μmol/L vs. below detection limit), but otherwise had an identical composition.

Patients attended the outpatient clinic at the start of the study period and at 4, 8, 12 and 16 weeks after inclusion. Blood and spent dialysate samples were obtained on each occasion.

Study patients

The study was approved by the ethics review board of the University of Heidelberg. Stable continuous ambulatory peritoneal dialysis (CAPD) patients were included after written informed consent. They were included if they were at least 18 years old, were treated with exchange volumes of 1500 to 2500 mL, and were able to handle a multicompartment fluid bag. Patients were excluded if they required antibiotic treatment, suffered from peritonitis, or were positive for hepatitis B, C or HIV. Overall 21 patients were randomly assigned to study group A ($N = 11$) or B ($N = 10$). Fifteen patients (9 and 6 patients in group A and B, respectively) completed the study. Reasons for drop out were peritonitis ($N = 4$), switch to hemodialysis ($N = 1$), pain upon instillation ($N = 1$, after changing from low GDP to conventional PD fluid). The mean age was 49 ± 12 years old in group A (3 males, 6 females) and 51 ± 8 years old in group B (5 males, 1 female). The time on CAPD was not significantly different (median 33 months, range 3 to 93 in group A vs. 10 months, range 2 to 30 in group B). Patients used 1.5% glucose, 2.5% glucose or 4% glucose containing PD fluids as required for the purpose of ultrafiltration.

Sampling of study material

Fifty milliliter samples of spent dialysate after short (2 hour) or overnight (8 hour) dwell were collected and immediately (<30 min) centrifuged at 2000 × g for 10 minutes before freezing the supernatant at −70°C for later analysis. Samples were thawed no more than once. The same procedure was adopted for the ethylenediaminetetraacetic acid (EDTA) blood samples.

Analytical techniques

Quantitation of methylglyoxal in PD fluid. Methylglyoxal was quantitated using a reverse phase C18 high-pressure liquid chromatography (HPLC) method according to Nilsson-Thorell et al [11]. The detection limit was 2.8 μmol/L.

Ex vivo generation of AGE-specific fluorescence in peritoneal dialysis fluid. The presence of reactive carbonyl compounds (i.e., GDP) in the peritoneal dialysis fluid was indirectly assessed by measuring the generation of AGE-specific fluorescence. Unused fluid and spent dialysate samples recovered after two or eight hours, respectively, were adjusted to a final glucose concentration of 1.5% by addition of GDP-free glucose stock solution (20% glucose in saline). The glucose concentration was measured in every sample of spent PD fluid; thereafter, nine parts of spent PD-fluid were supplemented with one part of the glucose stock solution and saline to yield 1.5% glucose as final concentration. Purified human serum albumin was added to a final concentration of 40 mg/mL (fraction V; Sigma Co., Taufkirchen, Germany). The latter was provided as a reactant for carbonyl compound mediated protein modification. The samples were sterile filtered (0.02 μm; Sarstedt Co., Nümbrecht, Germany) and incubated without any preservatives at 37°C for 3 and 10 days, respectively, at neutral pH. Thereafter, the AGE concentration was assessed as fluorescence intensity (excitation 350 nm/emission 430 nm) with a spectrofluorometer (LS 50 B; Perkin Elmer Co., Überlingen, Germany).

To complement the group-specific fluorescent assay with quantitative chemical measurements, the concentration of pyrraline as a non-fluorescent Amadori product was quantitated using HPLC after preceding enzymatic hydrolysis according to Henle et al [4].

Assessment of advanced oxidation protein products (AOPP). AOPP were measured according to Witko-Sarsat et al [12] using albumin as a reactant. The assay was calibrated with chloramine-T standard solutions (0 to

100 μmol/L) after addition of potassium iodide. Generation of AOPP was expressed as chloramine-T equivalents.

Quantitation of fluorescent compounds in plasma. Fluorescence intensity (exc. 350 nm/em. 430 nm) in plasma samples was quantified using a spectrofluorometer (LS 50 B; Perkin Elmer Co.). Fluorescence was also measured after size-selective separation of plasma protein fractions (size exclusion chromatography, Superdex 75 HR 10/30; Pharmacia Co., Freiburg, Germany) according to Henle et al [4]. The coefficient of variation for replicate measurements was <5%.

Quantitation of specific non-fluorescent AGE compounds in plasma. Carboxymethyllysine (CML) as a non-reactive AGE compound was analyzed in plasma using an enzyme-linked immunosorbent assay (ELISA; Roche Co., Mannheim, Germany) according to the manufacturer's instructions. Prior to the analysis enzymatic protein digestion was performed using proteinase K and phenylmethylsulfonyl fluoride (PMSF; Roche Co., Mannheim, Germany) according to the manufacturer's instructions. The intra-assay coefficient of variation was <5% and the interassay CV <10%. Fructoselysine was quantified by amino acid analysis using HPLC after prior acid hydrolysis [4].

Quantitation of cancer antigen 125 (CA 125). The glycoprotein CA 125 was analyzed in spent centrifuged dialysate using a commercially available ELISA (Roche Co.) according to the manufacturer's instructions.

Statistical analysis

Unless indicated otherwise, data are given as x ± SD. The selected study design, that is, two-group crossover, allows the effects of treatment modalities to be analyzed irrespective of sequence of therapy [13]. To exclude period and carry-over effects the data were analyzed according to Pocock [13], that is, separately in each study group calculating the mean of intra-individual differences and of individual patient data means obtained during the low and high GDP fluid periods. Mean values (patient data from group A vs. group B) were compared by applying the *t* test for unpaired data ($P < 0.05$ considered as significant).

After exclusion of period and carry-over effects, data from the two study groups could be pooled for calculation of means and standard deviation as well as for further statistical evaluation. Data were tested for normal distribution by the Wilk-Shapiro test.

T test for paired and unpaired samples were applied with normally distributed data for non-repeated measures.

Wilcoxon signed-rank test for paired data and Mann-Whitney test for unpaired data were used for data that were non-normally distributed (for non-repeated measures).

Repeated measures, for example, analyses at four and eight weeks, were analyzed using analysis of variance (ANOVA) for repeated measures after a check for normal distribution, by the Friedman test (for paired data) or Kruskal-Wallis test (for unpaired data).

Statistical evaluation was performed using data processing system RS/1 (V. 6.0.1; BBN Software Products Co., Cambridge, MA, USA).

RESULTS

Methylglyoxal in unused and spent PD fluid: Comparison of low GDP and conventional PD fluid

Unused conventional PD fluid contained 5.3 ± 1.6 μmol/L methylglyoxal, while in the unused low GDP PD fluid the methylglyoxal concentration was below the detection limit. In the 15 study patients, methylglyoxal concentration in the spent dialysate remained undetectable after the two and eight hour dwell during the phase with low GDP PD fluid. During the phase with conventional PD fluid, the concentration of methylglyoxal decreased rapidly and was no longer detectable (<2.8 μmol/L) after the two or eight hour dwell, respectively.

Analysis of AGE-specific fluorescence, pyrraline concentration and concentration of AOPP in the PD fluid after the dwell

Formation of AGE. As shown in Figure 1, when unused low GDP PD fluid was incubated with albumin as a reactant to generate carbonyl-mediated AGE compounds, very little increase in fluorescence (OD) as an overall index of AGE formation was noted (Fig. 1A). In contrast (Fig. 1B), when incubating unused conventional PD fluid, a marked increase in fluorescence was noted that was progressive with time of incubation. Generation of fluorescence was abrogated to a large extent when spent conventional dialysate that had been recovered after a two or eight hour dwell was used for the same experiment.

The above experiments were performed with 1.5% glucose-containing PD fluids. With 2.5% glucose-containing PD fluids, there was 54% higher generation of fluorescence (ΔOD 109 ± 6.9 vs. 70.6 ± 11.7), but this was not seen with the low GDP PD fluid.

Generation of pyrraline during incubation of low GDP and conventional PD fluids. When unused or spent PD fluid was incubated with albumin, generation of pyrraline, a non-fluorescent Amadori product, was significantly lower with low GDP compared to conventional PD fluid, as shown in Table 1. While the capacity to generate pyrraline during incubation with albumin ex vivo was not significantly modified when comparing unused fluid with spent dialysate after a two or eight hour dwell with low GDP PD fluid, a significant decrease was found with conventional PD fluid.

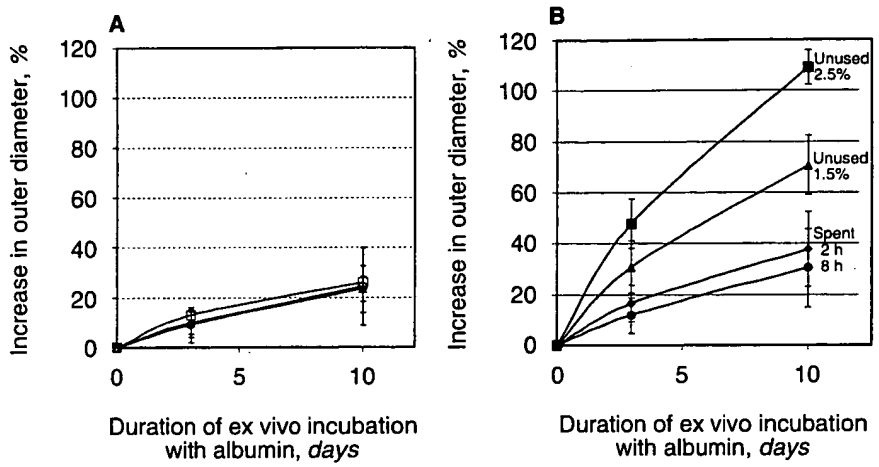


Fig. 1. Generation of fluorescent advanced glycation end products (AGE) compounds during incubation of low glucose degradation products (GDP) and conventional peritoneal dialysis (PD) fluids. The generation of fluorescent AGE compounds is given as the percent increase of optical density (excitation 350/emission 430 nm) of unused low GDP (*A*; $N = 6$ for 1.5%, $N = 6$ for 2.5% glucose) and conventional (*B*; $N = 6$ for 1.5%, $N = 6$ for 2.5% glucose) PD fluid and fluid recovered after a 2 hour [$N = 15$ in each group (low and high GDP)] and 8 hour [$N = 15$ in each group (low and high GDP] peritoneal dwell, respectively. Symbols are: (■) unused 2.5%; (▲) unused 1.5%; (◆) spent at 2 hours; (●) spent at 8 hours. For all corresponding comparisons, that is, unused fluids and fluids from different dwell times (2 vs. 8 hours) and ex vivo incubation times, respectively, generation of fluorescence was significantly lower with low GDP compared to conventional PD fluid ($P < 0.05$, paired data, Wilcoxon-test). These fluids contained 1.5% glucose.

Table 1. Generation of pyrraline (pmol/mg protein) during incubation of low GDP and conventional PD fluids with albumin as reactant

	Low GDP PD fluid	Conventional PD fluid
Unused fluid ($N = 6$)		
1.5% glucose	35.3 ± 18.2[a]	104.0 ± 28.1
2.5% glucose	41.2 ± 12.9[a]	120.2 ± 10.6
Spent dialysate ($N = 15$)		
2 hour dwell	33.7 ± 23.2[a]	40.5 ± 26.7[a]
8 hour dwell	41.1 ± 31.0[a]	35.2 ± 18.8[a]

Data are given as mean ± SD.
[a] $P < 0.01$ vs. unused conventional PD fluid (unpaired)

The build-up of pyrraline concentration in unused fluids was not significantly different when 1.5% and 2.5% glucose-containing fluids were used (Table 2).

Formation of AOPP. Advanced oxidation protein products were quantitated as chloramine-T equivalents in μmol/L. When unused PD fluids were analyzed, a significant increase of AOPP concentration was noted after 10 days of incubation versus baseline dependent on the glucose concentration ($P < 0.05$). The same was true for spent low GDP PD fluid recovered after two and eight hour dwells, respectively (Table 2).

Despite similar generation of AOPP by unused PD fluids at baseline, there was a significant increase of AOPP in spent dialysate with dwell time (8 vs. 2 hours, $P < 0.05$; Table 2).

Significantly higher AOPP levels of spent fluids versus unused PD fluids ($P < 0.05$) as well as the increase of AOPP levels over dwell period (8 vs. 2 hours) indicate that the major part of AOPP was formed during the intraperitoneal dwell. The finding of significantly higher AOPP concentrations at baseline as well as after 10 days ex vivo incubation of conventional PD fluids compared to low GDP fluids ($P < 0.05$) suggests GDP to be causally involved in the formation of AOPP.

Plasma fluorescence as an index of circulating AGE compounds: Comparison of the low GDP and conventional PD fluid periods

As shown in Figure 2, plasma fluorescence was significantly lower during the low GDP PD fluid period (22.1 ± 4.1 arbitrary fluorescence intensity units × 10^3) compared to the high GDP fluid period (24.4 ± 4.6 units × 10^3) after a treatment period of four weeks. The difference was unchanged after eight weeks in the low GDP or conventional PD fluid periods, respectively (data not given). In 10 controls, plasma fluorescence was 5.34 ± 1.1 and in 30 HD patients 19.9 ± 4.7 units × 10^3 (predialysis).

Figure 3 shows the molecular weight-specific analysis of fluorescent AGE species in plasma. The integrated fluorescence peaks according to molecular weight by size exclusion chromatography in the two study periods are given in the tables displayed in Figure 3.

A significant difference during the fourth week of the respective study period was noted between low GDP and conventional PD fluid: 533 ± 96.9 peak area (arbitrary units/10^4) on low GDP versus 576 ± 85.2 on conventional PD fluid (integrated fluorescence peaks, $P < 0.01$; Fig. 3). Representative chromatograms (patient R.P.) are given in Figure 3 to illustrate the method of peak-specific fluorescence analysis.

Quantitation of the non-fluorescent AGE compound carboxymethyllysine in plasma

As depicted in Figure 4, the plasma carboxymethyllysine concentration at baseline was 1.14 ± 0.44 mg/L. It

Table 2. Formation of advanced oxidized protein products (AOPP) (μmol/L) as chloramine-T equivalents at baseline and after 10 days of in vitro incubation with albumin

	Low GDP PD fluid			Conventional PD fluid		
	Baseline	10 days ex vivo incub.	Difference	Baseline	10 days ex vivo incub.	Difference
Unused fluid (N = 6)						
1.5% glucose	21.9 ± 1.1	26.2 ± 4.6	3.3 ± 4.2	24.7 ± 3.7	35.5 ± 4.6[a,d]	10.8 ± 4.9[a]
2.5% glucose	21.5 ± 0.9	31.3 ± 6.1[d]	9.8 ± 6.0	23.4 ± 1.6[a]	39.7 ± 6.4[a,d]	16.3 ± 6.7
Spent dialysate (N = 15)						
2-hour dwell	34.0 ± 5.4[b]	39.6 ± 5.5[b,d]	5.2 ± 1.9	36.5 ± 5.7[a,b]	45.4 ± 7.9[a,b,d]	9.3 ± 7.2
8-hour dwell	38.1 ± 6.7[b,c]	46.0 ± 7.6[b,c,d]	5.7 ± 2.2	41.7 ± 8.3[b,c]	49.7 ± 8.3[a,b,c,d]	7.9 ± 2.7

Data are given as mean ± SD.
[a] $P < 0.05$ vs. low GDP fluid (unpaired for unused PD-fluids, paired for spent PD-fluids)
[b] $P < 0.05$ vs. respective unused fluid (1.5% glucose) (unpaired)
[c] $P < 0.05$ vs. 2-hour dwell (paired)
[d] $P < 0.05$ vs. baseline (paired)

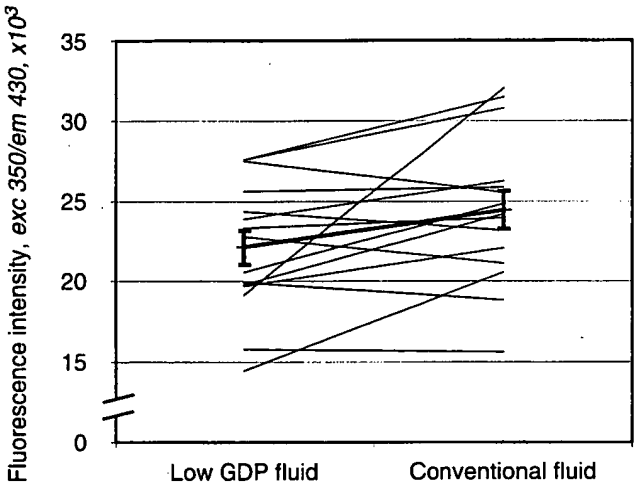


Fig. 2. Plasma fluorescence during the low GDP and conventional PD fluid periods. Plasma fluorescence (OD 350/430 nm arbitrary units × 10^3) is shown after a low GDP PD fluid period of 4 weeks (median 22.8) and after a conventional PD fluid period of 4 weeks (median 24.2). Data are given as individual patient data and as mean ± SEM. The difference was statistically significant ($P < 0.05$ Wilcoxon test for paired data).

was significantly lower after eight weeks of treatment with the low GDP PD fluid (1.12 ± 0.47) compared to eight weeks of treatment with the high GDP PD fluid (1.24 ± 0.44; $P < 0.05$).

The change in CML concentration is not explained as an artifact of hemoconcentration based on plasma albumin (baseline 39.5 ± 3.2 g/L; 8 weeks 38.6 ± 4.4) and hematocrit (Hct) measurements (baseline 33.5 ± 3.8%; 8 weeks 34.9 ± 4.0%).

Plasma fructoselysine levels did not show any significant change during the low GDP and conventional PD fluid periods (40.7 ± 7.9 vs. 40.0 ± 8.0 mg/L). The concentrations in controls were 5.1 ± 1.95 mg/L and in hemodialysis patients 1.61 ± 0.5 mg/L.

Glycoprotein CA 125 concentration in supernatants of spent PD fluids

As shown in Table 3, baseline glycoprotein CA 125 concentrations in the cell-free supernatant of spent peritoneal dialysates were low, but they were significantly higher at the end of the low GDP PD fluid period. In the group that was first exposed to conventional and subsequently to low GDP fluid, a rapid recovery was noted after four weeks of low GDP fluid with no further increase at eight weeks (Table 3). Conversely, in the group where after eight weeks of treatment with low GDP fluid the glycoprotein CA 125 concentration was 30.9 ± 5.3 U/mL, the concentration decreased within four weeks on conventional PD fluid to 8.9 ± 2.2 U/mL ($P < 0.05$). These data refer to the eight hour dwell. The data profile was similar but approximately 30% lower when spent dialysate of the two hour dwell was analyzed (data not shown).

DISCUSSION

The above data clearly show that PD fluids with high concentrations of glucose degradation products (GDP) generated during heat sterilization at an acidic pH are rapidly modified during two hours and particularly at eight hours of intraperitoneal dwell. The data further suggest that GDP disappear from the PD fluid during the dwell. This conclusion is based on measurements of methylglyoxal as one representative GDP compound that was no longer demonstrable after a two hour dwell. Furthermore, progressive loss of the capacity to form AGE by ex vivo incubation with albumin as a reactant suggests a rapid and progressive decrease of the concentration of carbonyl compounds during a peritoneal dwell. The study does not permit a decision about whether this disappearance reflects spontaneous decay, interaction with intraperitoneal molecules, particularly proteins, interaction with the peritoneal wall or absorption into the systemic circulation (or a combination of these possibilities). At any rate, the observation of higher fluorescence in the plasma during the period when conventional PD fluids were used is compatible with the idea that carbonyl precursors, or AGE as their product, enter the systemic circulation. It has not been documented, and is unlikely,

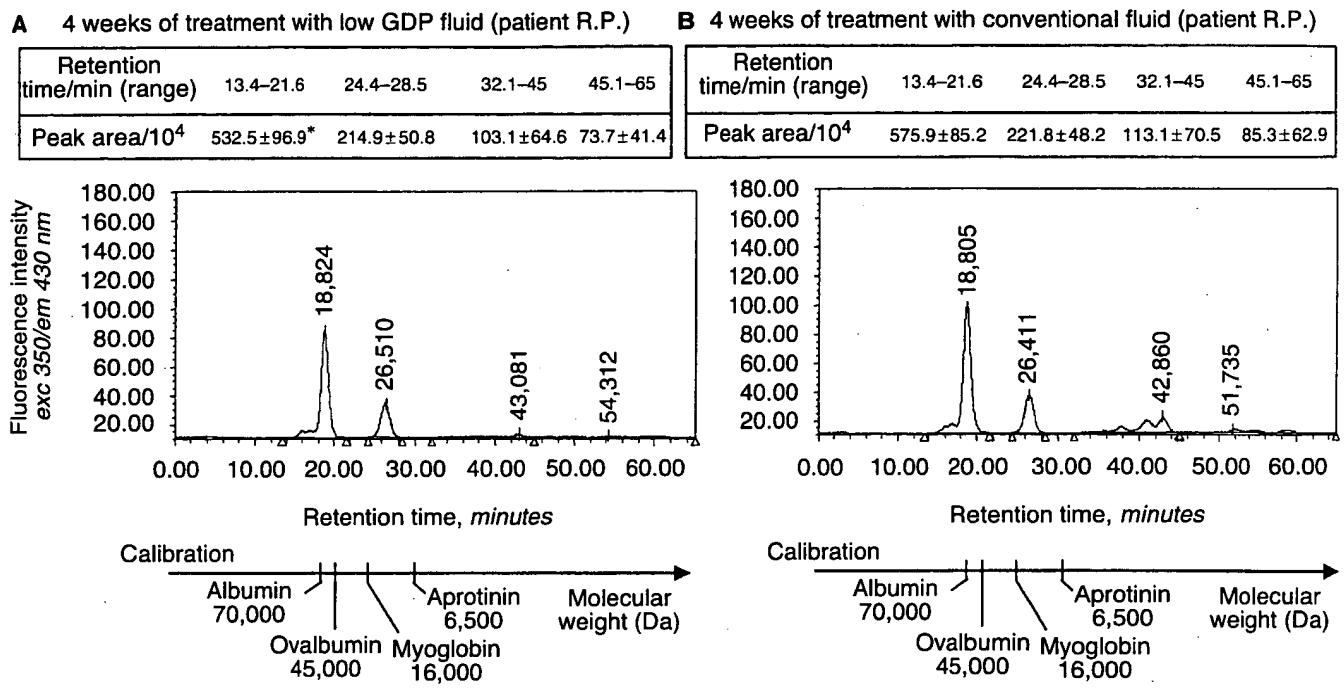


Fig. 3. Molecular weight-specific analysis of fluorescent AGE in plasma shows representative characteristic plasma fluorescence peak patterns of patient R.P. after 4 weeks treatment with low GDP PD fluid (*A*) and of the same patient after 4 weeks treatment with conventional PD fluid (*B*). Tables displayed at the top of the panels give the values for the mean peak areas ± SD of all patients obtained after the respective study periods. A significant difference (*$P < 0.05$, paired data, Wilcoxon test) was found for the integrated peak area of the high-molecular-weight fraction (that is, retention time 18.8 seconds corresponding to albumin as shown by independent calibration samples).

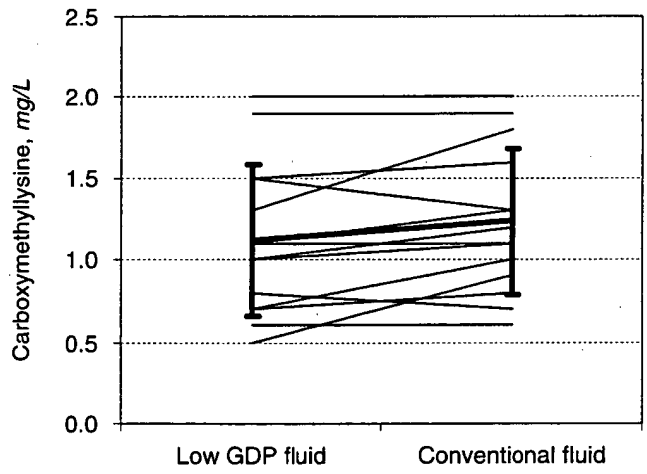


Fig. 4. Plasma carboxymethyllysine concentration during the low GDP and conventional PD fluid periods. The concentration of carboxymethyllysine in plasma is seen after a low GDP PD fluid period and after a conventional PD fluid period of 8 weeks each. Data are given as individual patient data and as mean ± SD. The difference was statistically significant ($P < 0.05$) using the *t* test for paired data.

Table 3. Glycoprotein CA 125 (U/mL) in supernatants of spent dialysates during the low GDP and conventional PD fluid treatment periods ($N = 15$)

	Low GDP fluid		Conventional fluid	
Start of study	4 weeks	8 weeks	4 weeks	8 weeks
13.5 ± 3.1	33.4 ± 3.7[a]	31.8 ± 4.1[a]	14.4 ± 3.6	11.8 ± 1.5

Data are given as mean ± SEM.
[a] $P < 0.05$ vs. start and conventional fluid (Wilcoxon-Wilcox)

that the highly reactive GDP bind to plasma protein and are transferred intact into the plasma compartment via lymphatic absorption. The validity of the fluorescence assay is reinforced by measurements of the specific Amadori product CML, which yielded the same result. A reverse transport of carbonyl compounds from the plasma space into the peritoneal fluid, as postulated by others, is not demonstrated in the present study. The study confirms previous observations [7, 8] to the extent that the glycoprotein CA 125, an index of mesothelial cell number and/or viability, is beneficially affected by low GDP PD fluid. One would assume that highly reactive carbonyl compounds in the peritoneal cavity trigger inflammatory processes possibly mediated via nuclear factor-κB (NF-κB) [14] or protein kinases, but we could not show changes in the plasma concentration of C-reactive protein (CRP) or other markers of microinflammation during the low GDP PD fluid period (data not shown). The periods may have been too short, however, to demonstrate such a benefit.

Several methodological points of the study deserve further comment. The high patient attrition rates on CAPD,

noticed also in previous studies [8], creates definite difficulties as reflected by the fact that only 15 of the 21 recruited patients completed the 16 week study. A further problem is the large inter-patient variability. For this reason, we used a prospective randomized crossover design to deal with interindividual differences. The shortcoming of this design is that the duration of the study was restricted to the eight-week intervention period, limiting the possibility to observe a true long-term benefit from the study fluid. This shortcoming was more than balanced, however, by the fact that patients could be taken as their own controls. The consistency of the observation was improved by repeat measurements at four and eight weeks during the study periods. Measurements were highly congruent at four and at eight weeks, respectively. We also emphasize that all measurements were carried out at least in duplicate. There is uncertainty whether CA 125 really reflects mesothelial cell number or mesothelial cell viability. The rapid normalization (and the reversibility thereof) are more in favor of the latter possibility. This notion is also supported by the observation that the changes in CA 125 occurred very rapidly, for example, in non-systematic studies within two weeks time [15]. A possible interference of GDP with the CA 125 measurements was excluded by appropriate controls.

The measurements of AGE and AOPP were performed as group-specific global tests to assess carbonyl stress and accumulated oxidative stress in the effluent fluid, respectively. As far as the AGE assay is concerned, we emphasize that the incubation was carried out without additives, particularly without antibiotics, and in contrast to other studies on this topic, pH was monitored to detect potential glucose degradation and generation of products impacting on pH. Because of the non-specificity of the fluorescence test for AGE, we additionally measured the concentration of the early Amadori product pyrraline that is generated by the interaction of 3-deoxyglucosone with α-amino groups in general or ε-amino groups of lysine [4]. Fluorescence and pyrraline concentrations increased in parallel (abstract; Zeier et al, *J Am Soc Nephrol* 12:317A, 2001). In parallel with the decrease of the concentration of the AGE precursor methylglyoxal, the capacity to generate AGE decreased rapidly during the intraperitoneal dwell. In contrast AOPP, that is, the long-lived products of oxidative damage assessed as chloramine-T equivalents, increased rapidly [12]. The rapid generation of AOPP during short intraperitoneal dwells presumably reflects high intraperitoneal oxidative stress, possibly mediated by carbonyl compounds. The AGE and AOPP measurements ex vivo, reflecting the capacity to glycate and oxidize, respectively, are analogous to the carbonyl test proposed by Miyata et al [16].

Carbonyl compounds disappear during the intraperitoneal dwell and we wondered about the fate of these compounds. We are aware of the limitations of the measurements of plasma fluorescence as an index of AGE compounds. This global assay is susceptible to numerous artifacts. We carefully excluded artifacts from lipemia. Patients were without infection. The only medications administered were phosphate binders, vitamin D preparations, and antihypertensive agents. The dose of antihypertensive agents, particularly angiotensin-converting enzyme (ACE) inhibitors, was not changed between the two investigation periods. We are aware of the possibility that the plasma AGE concentration may be affected by AGE compounds in the diet [17]. Therefore, the patients were advised not to change the intake of nutrients with known high AGE content. The small increase in plasma fluorescence was highly significant in the intra-individual comparisons and points to the systemic absorption of GDP, of Amadori products or AGE compounds, respectively. The validity of the measurement is further supported by the observation by an independent analytical method: the fluorescence was specifically increased in a subfraction with the molecular weight of albumin. Therefore, a confounding effect by fluorescent low molecular weight compounds can be excluded, a point of interest because these fractions are specifically increased in uremia as shown by Henle et al [4]. The results of the relatively non-specific fluorescence are confirmed by the parallel increase in the chemically defined compound CML.

What are the potential long-term consequences of our observation?

In the past it was always assumed that high glucose was the major culprit in the genesis of peritoneal membrane damage. Deterioration of peritoneal membrane characteristics is to a large extent associated with new vessel formation, that is, angiogenesis [18]. In this context it is of interest that GDP were shown to stimulate angiogenesis directly independent of glucose [19]. De Vriese et al documented a role of glucose in the development of peritoneal angiogenesis that was mediated by vascular endothelial growth factor (VEGF) [20]. The relative roles of glucose and GDP (as well as their potential synergism) in the stimulation of peritoneal neoangiogenesis require further study. Furthermore, there are numerous possibilities other than neoangiogenesis for how the highly reactive carbonyl compounds (GDP) might damage the peritoneal membrane.

Our observations on the deleterious potential of GDP go beyond damage to the peritoneal membrane, however, and are even consistent with the possibility of systemic effects following absorption of intraperitoneal GDP (or compounds derived thereof). This finding is of considerable concern. Uremic toxicity has recently been ascribed, at least in part, to AGE formation [21]. A contribution of the PD fluid to systemic accumulation of AGE, although relatively small in our short-term study, therefore may not be negligible. This possibility was pre-

viously entertained by other authors who speculated that in addition to the carbonyl load resulting from uremia, glucose-derived reactive carbonyl compounds may be involved in the functional alteration of the peritoneal membrane [22]. Furthermore, Witowski et al studied long-term human peritoneal mesothelial cell cultures and noted that glucose degradation products impair viability, interleukin-6 (IL-6) secretion as well as fibronectin release [23].

Finally, in a prospective randomized study, Jones et al documented that the addition of bicarbonate modulates concentrations of putative markers of peritoneal membrane integrity and inflammation [24], a point that was not addressed in the present study.

In summary, our study results lend further support to the postulate [25] that formation of GDP during sterilization and storage of PD fluid should be avoided.

Reprint requests to Professor Dr. Dr. h.c. mult. Eberhard Ritz, Department of Internal Medicine, University of Heidelberg, Bergheimer Straße 58, D-69115 Heidelberg, Germany.
E-mail: prof.e.ritz@t-online.de

REFERENCES

1. Martinson E, Wieslander A, Kjellstrand P, Boberg U: Toxicity of heat sterilized peritoneal dialysis fluids is derived from degradation of glucose. *ASAIO J* 38:M370–M372, 1992
2. Wieslander A, Andrén A, Martinson E, *et al*: Toxicity of effluent peritoneal dialysis fluid. *Adv Perit Dial* 9:31–35, 1993
3. Maillard LC: Action des amides aminés sur les sucres. Formation des mélanoides par voi méthodique. *C R Acad Sci Ser* 145:66–68, 1912
4. Henle T, Deppisch R, Beck W, *et al*: Advanced glycated endproducts (AGE) during haemodialysis treatment: Discrepant results with different methodologies reflecting the heterogeneity of AGE compounds. *Nephrol Dial Transplant* 14:1968–1975, 1999
5. Wieslander A, Forsbäck G, Svensson E, *et al*: Cytotoxicity, pH, and glucose degradation products in four different brands of PD fluids. *Adv Perit Dial* 12:57–60, 1996
6. Lindén T, Forsbäck G, Deppisch R, *et al*: 3-deoxyglucosone, a promoter of advanced glycation end products in fluids for peritoneal dialysis. *Perit Dial Int* 18:290–293, 1998
7. Cappelli G, Bandiani G, Cancarini GC, *et al*: Low concentrations of glucose degradation products and their impact on biocompatibility parameters: Prospective cross-over study with a three-compartment bag. *Adv Perit Dial* 15:238–242, 1999
8. Rippe B, Simonsen O, Heimbürger O, *et al*: Long-term clinical effects of a peritoneal dialysis fluid with less glucose degradation products. *Kidney Int* 59:348–357, 2001
9. Koomen GDM, Betjes MGH, Zemel D, *et al*: Cancer antigen 125 is locally produced in the peritoneal cavity during continuous ambulatory peritoneal dialysis. *Perit Dial Int* 14:132–136, 1994
10. Visser CE, Brouwer-Steenbergen JJE, Betjes MGH, *et al*: Cancer antigen 125: A bulk marker for the mesothelial mass in stable peritoneal dialysis patients. *Nephrol Dial Transplant* 10:64–69, 1995
11. Nilsson-Thorell CB, Muscalu N, Andrén A, *et al*: Heat sterilization of fluids for peritoneal dialysis gives rise to aldehydes. *Perit Dial Int* 13:208–213, 1993
12. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, *et al*: Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 49:1304–1313, 1996
13. Pocock SJ: The analysis and interpretation of crossover trials, in *Clinical Trials*, Chichester, John Wiley & Sons, 1983, pp 114–119
14. Kislinger T, Fu C, Huber B, *et al*: N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem* 274:31740–31749, 1999
15. Hylander B, Granroth B, Wieslander A: CA 125 during the first 8 weeks of CAPD treatment: Influence of GDP. *Perit Dial Int* 21(Suppl 1):27, 2001
16. Miyata T, Horie K, Ueda Y, *et al*: Advanced glycation and lipidoxidation of the peritoneal membrane: Respective roles of serum and peritoneal fluid reactive carbonyl compounds. *Kidney Int* 58:425–435, 2000
17. Koschinsky T, He CJ, Mitsuhashi T, *et al*: Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci USA* 94:6474–6479, 1997
18. De Vriese AS, Tilton RG, Stephan CC, Lameire NH: Vascular endothelial growth factor is essential for hyperglycemia-induced structural and functional alterations of the peritoneal membrane. *J Am Soc Nephrol* 12:1734–1741, 2001
19. Inagi R, Miyata T, Yamamoto T, *et al*: Glucose degradation product methylglyoxal enhances the production of vascular endothelial growth factor in peritoneal cells: Role in the functional and morphological alterations of peritoneal membranes in peritoneal dialysis. *FEBS Lett* 463:260–264, 1999
20. De Vriese AS, Mortier S, Lameire NH: Glucotoxicity of the peritoneal membrane: The case for VEGF. *Nephrol Dial Transplant* 16:2299–2303, 2001
21. Miyata T, Sugiyama S, Saito A, Kurokawa K: Reactive carbonyl compounds related uremic toxicity ("carbonyl stress"). *Kidney Int* 59(Suppl 78): S25–S31, 2001
22. Miyata T, Devuyst O, Kurokawa K, van Ypersele de Strihou C: Toward better dialysis compatibility: Advances in the biochemistry and pathophysiology of the peritoneal membranes. *Kidney Int* 61: 375–386, 2002
23. Witowski J, Wisniewska J, Korybalska K, *et al*: Prolonged exposure to glucose degradation products impairs viability and function of human peritoneal mesothelial cells. *J Am Soc Nephrol* 12:2434–2441, 2001
24. Jones S, Holmes CJ, Krediet RT, *et al*: Bicarbonate/lactate-based peritoneal dialysis solution increases cancer antigen 125 and decreases hyaluronic acid levels. *Kidney Int* 59:1529–1538, 2001
25. Wieslander AP: Cytotoxicity of peritoneal dialysis fluids – is it related to glucose breakdown products? *Nephrol Dial Transplant* 11:958–959, 1996

Exhibit M

FERTILITY AND STERILITY®
VOL. 76, NO. 6, DECEMBER 2001

Published by Elsevier Science Inc.
Printed on acid-free paper in U.S.A.

Expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in serosal tissue of intraperitoneal organs and adhesions

Nasser Chegini, Ph.D.,[a] Kristina Kotseos, B.Sc.,[a] Yong Zhao, M.D.,[a] Chunfeng Ma, M.D., Ph.D.,[a] Frederick McLean, M.D.,[a] Michael P. Diamond, M.D.,[b] Lena Holmdahl, M.D., Ph.D.,[c] James Burns, Ph.D.,[d] and The Peritoneal Healing and Adhesion Multiuniversity Study (PHAMUS) Group

Department of Obstetrics and Gynecology, Institute for Wound Research, University of Florida, Gainesville, Florida

Objective: To compare expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in serosal tissue of intraperitoneal organs and adhesions.

Design: Prospective and cross-sectional study.

Setting: Academic research centers.

Patient(s): Patients undergoing abdominal or pelvic surgery.

Intervention(s): MMP-1 and TIMP-1 expression.

Main Outcome Measure(s): Expression of messenger ribonucleic acid (mRNA) and protein was measured by using quantitative reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay.

Result(s): Serosal tissue of intraperitoneal organs and adhesions express MMP-1 and TIMP-1 mRNA and protein at levels that are consistently varied with 10- to 10,000-fold and 2- to 10-fold higher TIMP, mRNA and protein, respectively. Parietal peritoneum, fallopian tubes and ovaries express higher MMP-1 mRNA levels compared with uterus and adhesions; the lowest expression is found in small and large bowels, subcutaneous tissue. and omentum. Expression of TIMP-1 mRNA was less variable; the highest level was found in the uterus and the lowest in subcutaneous tissue and small bowels. There was less variability in MMP-1 and TIMP-1 protein content than mRNA expression; ovaries and adhesions contained the highest MMP-1 and TIMP-1 levels, respectively, and peritoneum contained the lowest. The MMP-1 and TIMP-1 content and ratios further indicate limited MMP-1 proteolytic activity. Although tissues from premenopausal women express more MMP-1 and TIMP-1, expression did not differ by sex or age.

Conclusion(s): Because MMP-1 and TIMP-1 expression varies consistently among the serosal tissues of peritoneal organs and adhesions, and because tissue injury alters their expression, site-specific variations in expression of these substances may predispose a particular organ to develop more adhesions. (Fertil Steril® 2001;76:1212–9. ©2001 by American Society for Reproductive Medicine.)

Key Words: MMP-1, TIMP-1, mRNA, protein, serosal tissue, peritoneal adhesion

Received December 7, 2000; revised and accepted July 6, 2001.

Supported by Genzyme Corp., Cambridge, Massachusetts.

Presented in part at the 54th Annual Meeting of the American Society for Reproductive Medicine, San Francisco, California, 1998, and selected as the Prize Paper by the Society for the Reproductive Surgeons.

Reprint requests: Nasser Chegini, Ph.D., Department of Obstetrics and Gynecology, University of Florida, Box 100294, Gainesville, Florida 32610-0294 (FAX: 352-392-6994; E-mail: cheginin@obgyn.ufl.edu).

[a] Department of Obstetrics and Gynecology, University of Florida, Gainesville, Florida.

[b] Department of Obstetrics and Gynecology, Wayne State University, Detroit, Michigan.

[c] Department of Surgery, Gothenberg University, Gothenberg, Sweden.

[d] Genzyme Corporation, Cambridge, Massachusetts.

0015-0282/01/$20.00
PII S0015-0282(01)02875-8

The serosal surface of the parietal peritoneum and of the organs within the peritoneal cavity consists of a layer of mesothelial cells that prevent the peritoneal organs from adhering to the surfaces opposite them. Extensive cellular or tissue injury to the serosal tissue after surgical procedures or as a result of peritoneal infection and inflammation alters the healing processes that often leads to development of peritoneal adhesions.

Although these adhesions develop in many patients after pelvic or abdominal surgery, it is poorly understood why adhesions form more frequently in one tissue or patient than in others (1–3). A recent epidemiologic study indicated that the rate of hospital readmissions because of peritoneal adhesions is higher among women who undergo gynecologic surgeries than in those undergoing other pelvic or abdominal surgery (3).

Unregulated wound healing processes affecting cellular migration and proliferation and tissue remodeling result in formation of scar tissue such as peritoneal adhesions. These processes are highly regulated by the local action of various molecules, including several endoproteases with a broad range of enzyme activity (4, 5). Matrix metalloproteinases (MMPs) are a key member of the endoprotease family and are classified according to their substrate specificity—for example, MMP-1, which degrades collagens I–III and VII and fibronectin (4, 5). Under normal conditions the expression of MMPs is limited to a few tissues that undergo extensive remodeling, such as endometrium during the menstrual cycle, wound during healing, and inflammatory reactions (5–12).

The expression of MMPs is highly regulated at the transcriptional level and is influenced by local action of various cytokines, growth factors, and hormones (5–12). Moreover, MMPs are secreted as proenzymes that require activation to exert their biological activities, which include cell migration, angiogenesis, proliferation, and tissue remodeling. By releasing the growth factors and cytokines stored in the extracellular matrix, MMPs influence other cellular activities (4–6).

The proteolytic activity of the MMPs is regulated in part by their physiologic inhibitors, tissue inhibitors of MMPs (TIMPs), which consist of TIMP-1 to TIMP-4 (12, 13). In contrast to MMPs, TIMPs are expressed in a wide variety of tissues. In addition to inhibiting the proteolytic activities of MMPs, TIMPs regulate cell migration, angiogenesis, and cell growth (5, 6, 13–16). Several cytokines, growth factors, and hormones have also been shown to regulate expression of TIMPs (5, 7, 12, 13). Because MMPs and TIMPs are key components of the above processes, their coordinated and balanced expression is considered critical for normal wound healing (4–6, 17–22).

Studies of the expression of MMPs and TIMPs in the peritoneal environment have demonstrated their presence in peritoneal fluid of surgically induced adhesions in rats (23) and their expression in peritoneal serosal tissue, mesothelial cells, and peritoneal fluid of patients with and without adhesions (24–28). These findings suggest a potential role for MMPs and TIMPs in peritoneal wound healing and adhesion formation. On the basis of the knowledge that tissue injury alters expression of various molecules, including MMPs and TIMPs, consistent variations in local expression of these substances in serosal tissue of peritoneal organs may predispose an organ to develop more adhesions than others.

We sought to examine whether the serosal tissue of intraperitoneal organs and adhesions express MMP-1 and TIMP-1 mRNA and protein and whether the degree of their expression varies consistently between different organs, including parietal peritoneum, uterus, oviducts, ovaries, and large and small bowels. We also examined expression of these substances in the omentum and adhesions of patients with and without adhesions. Expression of these substances in skin, fascia, and subcutaneous tissue obtained from the same patients was also measured.

MATERIALS AND METHODS

All materials for isolation of total RNA, reverse transcription polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) were purchased from commercial sources, as described elsewhere (28–30). Human-specific MMP-1, TIMP-1 and MMP-1/TIMP-1 complex ELISA kits with limits of detection of 1.7, 1.3, and 1.5 ng/mL, respectively, were purchased from Oncogene Sciences, Inc. (Cambridge, MA).

Samples of skin (n = 34), fascia (n = 8), subcutaneous tissue (n = 12), omentum (n = 10), ovary (n = 5), and serosal tissue of the parietal peritoneum (n = 33), uterus (n = 22), fallopian tubes (n = 7), large and small bowel (n = 14), and adhesions (n = 16) were collected from 57 patients who were undergoing abdominal or pelvic surgery. Of these patients, 45 were female and 12 were male; the age range was 24 to 83 years. Thirty-two of the female patients were premenopausal, and 23 of these women had undergone previous surgical procedures, including cesarean section, bilateral tubal ligation, appendectomy, ovarian cystectomy, hysterectomy, and treatment for endometriosis. According to the date of last menstrual period and endometrial histology, 9 premenopausal patients were in the proliferative phase and 23 were in the secretory phase of the menstrual cycle. Thirteen women were postmenopausal. Male patients were scheduled to undergo various gastrointestinal surgical procedures.

Pelvic findings at surgery were used to assess the type of adhesions, which were classified on the basis of their severity as described elsewhere (31). These specimens were collected from patients undergoing surgical procedures at the University of Florida, Wayne State University, and Gothenburg University, after standardization of tissue handling and shipments. Approval from the institutional review boards at each institution was obtained before initiation of the study. All patients gave informed written consent before undergoing surgery.

After collection, the tissue pieces were divided into multiple portions and were either snap-frozen and stored in liquid nitrogen for mRNA and protein analysis or fixed for histologic evaluation. All samples for mRNA isolation, protein extraction, and histologic evaluation were centrally processed and analyzed at the University of Florida. For mRNA analysis, total cellular RNA was isolated from each tissue and subjected to quantitative RT-PCR using an external synthetic complementary RNA (cRNA) standard to determine MMP-1 and TIMP-1 mRNA expression, as described elsewhere (28–30).

The procedure used to construct the synthetic template for quantitative RT-PCR has been described in detail elsewhere

FIGURE 1

Competitive quantitative reverse transcriptase polymerase chain reaction (PCR) analysis of matrix metalloproteinase (*MMP-1*) and tissue inhibitor of MMP-1 (*TIMP-1*) messenger RNA using total cellular RNA isolated from parietal peritoneum. The upper bands are the PCR products generated from the specific message in cellular RNA, and the lower bands are from the standard complementary RNA (shown from right to left at dilutions corresponding to 10^8 to 10^3 and 10^7 to 10^2 copies/reaction). M = DNA markers.

Chegini. MMP-1 and TIMP-1 in serosal tissue. Fertil Steril 2001.

(29). The cDNA was synthesized in a series of standard reactions, each of which contained 2 μg of total cellular RNA from each tissue and several dilutions of cRNA (10^2 to 10^8 copies/reaction). The PCR products were separated on 2% agarose gels containing ethidium bromide, and the images were captured on a Kodak 120 digital camera (Eastman Kodak Co., Rochester, NY) and stored as a TIFF file on the computer. The PCR band intensities were determined and normalized for their molecular weight, and their ratio was plotted against the copy number of cRNA standard per reaction, as described elsewhere (28–30). The level of mRNA expression was determined if the ratio of cRNA to target band intensities was 1; analysis by equation of best-fit lines was performed, and results were reported as the mean ± SE of copies mRNA/μg total RNA (28–30).

To determine levels of MMP-1, TIMP-1, and MMP-1/TIMP-1 complex proteins, an equal amount of tissue was homogenized in homogenizing buffer, as described in the protocol of the ELISA kits. The homogenates were centrifuged at 4000 ×*g* for 15 minutes, and the supernatants were collected, divided into aliquot, and stored at −80°C until assay. The total protein content of the tissue extracts was determined by using a standard protein assay (Bio-Rad, Hercules, CA).

An equal amount of total protein from each preparation was subjected to ELISA measuring total MMP-1 (free and complex with TIMP-1), total TIMP-1 (free and complex with MMPs), and MMP-1/TIMP-1 complex (activated MMP-1 in complex with TIMP-1), respectively. All measurements were performed in duplicate, and results are reported as mean ± SE/mg of total protein.

The nonparametric Student *t*-test and Kruskal–Wallis one-way analysis of variance with the Dunn test were done by using SigmaStat software (Jandel Co., San Rafael, CA). A probability level of $P<.05$ was considered significant.

RESULTS

Standard RT-PCR indicated that all tissues examined in our study expressed MMP-1 and TIMP-1 mRNA (data not shown). Quantitative RT-PCR was performed to determine the level of MMP-1 and TIMP-1 mRNA expression in these tissues, using a competitive cRNA as internal standard. An example of quantitative RT-PCR using total cellular RNA isolated from parietal peritoneum and several dilutions of the cRNA standard is shown in Figure 1. Results of quantitative RT-PCR indicated that the level of MMP-1 and TIMP-1 mRNA expression varied substantially among these tissues; expression of TIMP-1 was 10- to 10,000-fold higher than expression of MMP-1 (Fig. 2).

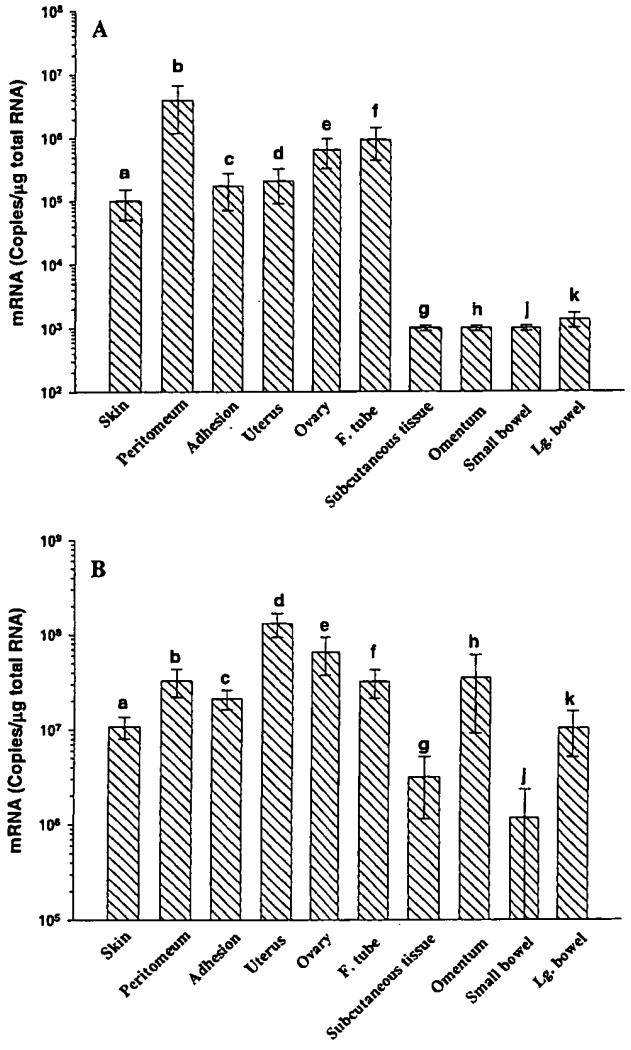
We found that expression of MMP-1 mRNA was greater in the parietal peritoneum than in uterus and adhesions ($P<.05$) but not in ovaries and fallopian tubes (Fig. 2A); however, MMP-1 mRNA expression was significantly higher in all of the above tissues compared with large and small bowels, omentum, and subcutaneous tissues ($P<.001$; Fig. 2A). The level of MMP-1 mRNA expression was lower in skin than in parietal peritoneum ($P<.05$) but not adhesions (Fig. 2A).

Uterine serosal tissue expressed more TIMP-1 mRNA than did parietal peritoneum, fallopian tubes, large bowel, and adhesions ($P<.05$) but not ovaries and omentum (Fig. 2B). Small bowel and subcutaneous tissues expressed the least TIMP-1 mRNA, whereas expression in skin was similar to that in adhesions and parietal peritoneum (Fig. 2B). Adhesions expressed approximately 100-fold more TIMP-1 mRNA than MMP-1 mRNA ($P<.01$) (Fig. 2).

Tissue extracts contained MMP-1 and TIMP-1 protein with varying levels ranging from 2- to 10-fold (Fig. 3A and 3B). Fallopian tubes and ovaries contained higher MMP-1 levels, followed by large bowel, uterus, omentum, adhesions, parietal peritoneum, fascia, and skin. Levels of MMP-1 protein were significantly higher in uterus, ovary, fallopian tube, and large bowel than in other tissues ($P=.005$, .003, and .01, respectively). Large bowel, uterus, and ovaries contain higher levels of TIMP-1 protein, followed by adhesions, fallopian tube, parietal peritoneum, omentum, skin, and fascia. These levels were significantly higher in adhesions, uterus, ovary, fallopian tube and large bowel than in

FIGURE 2

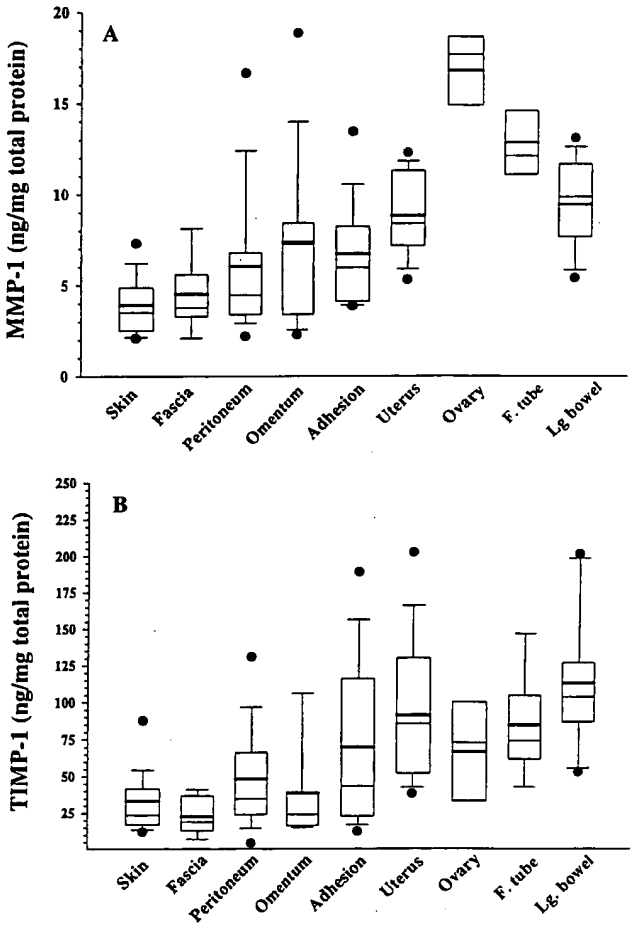
Expression of matrix metalloproteinase (**A**) and tissue inhibitor of MMP-1 (**B**) messenger RNA (*mRNA*) expression in serosal tissue of parietal peritoneum, various peritoneal organs, adhesions, and skin. Values were calculated from the band densities described in Materials and Methods. Results are means ± SE of copies of mRNA/μg total RNA. In panel A, $P<.05$ for b compared with a, c, and d; $P=.01$ for b compared with g, h, j, and k; and $P<.01$ for a, b, c, d, e, and f compared with g, h, j, and k. In panel B, $P<.05$ for d compared with a, b, c, f, g, j, and k.



Chegini. MMP-1 and TIMP-1 in serosal tissue. Fertil Steril 2001.

FIGURE 3

Notched box plots showing the level of matrix metalloproteinase (*MMP-1*) (**A**) and tissue inhibitor of MMP-1 (*TIMP-1*) (**B**) in samples of serosal tissue of the ovaries, uterus, fallopian tubes (*F. tube*), large bowel, omentum, skin, fascia, and adhesions. The vertical line within the box boundaries represent the distribution of the middle 50%, the thin horizontal lines within the boxes are the medians, and the thick lines are the arithmetric means. The notches represent the 95th percentile, and the error bars represent the 90th and 10th percentiles. Levels of MMP-1 levels in skin, fascia, peritoneum and omentum were significantly lower than those in uterus, ovary, fallopian tube, and large bowel ($P=.005$, .003, and .01, respectively), and TIMP-1 levels in skin and fascia were significantly lower than those in uterus, ovary, fallopian tube, and large bowel ($P=.002$, .05 and .0004, respectively).



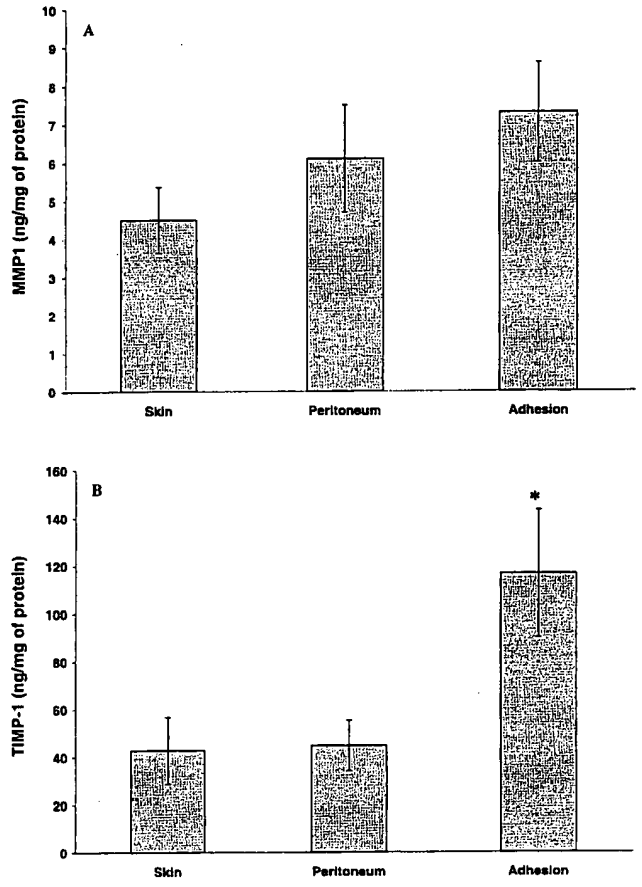
Chegini. MMP-1 and TIMP-1 in serosal tissue. Fertil Steril 2001.

other tissues ($P=.002$, .05, and .0004, respectively); comparison with levels in parietal peritoneum, adhesions, and ovary was not statistically significant. Comparative analysis of MMP-1 and TIMP-1 protein content in adhesions with intact parietal peritoneum and skin from the same patient further indicated substantial variation in their content among patients (data not shown).

In patients with adhesion, the tissues contained significantly more TIMP-1 compared with peritoneum and skin ($P=.05$ and .03) (Fig. 4A and 4B). Because MMPs are

FIGURE 4

Mean ± SE matrix metalloproteinase (*MMP-1*) (**A**) and tissue inhibitor of MMP-1 (*TIMP-1*) (**B**) protein content in adhesions, intact parietal peritoneum, and skin of 10 patients with adhesions. The TIMP-1 protein content in adhesions is significantly higher than that in intact parietal peritoneum ($P=.05$) and skin ($P=.03$).



Chegini. MMP-1 and TIMP-1 in serosal tissue. Fertil Steril 2001.

produced as proenzymes and bind to TIMPs immediately after activation, we measured levels of MMP-1/TIMP-1 complex and ratios of MMP-1 to TIMP-1 in tissue extracts. The amount of MMP-1/TIMP-1 complex in adhesions was relatively low compared with skin, fascia, and peritoneum (Fig. 5A); these latter tissues in turn contained significantly lower levels than did other tissues ($P < .001$). The levels of MMP-1/TIMP-1 complex in adhesions differed significantly from those in uterus and ovary ($P=.03$ and .001). In addition, 65% to 37% of MMP-1 detected in the tissue extracts appeared to be in complex with TIMPs (Fig. 5B). The ratio of MMP-1 to TIMP-1 was significantly lower in adhesions than in peritoneum and skin from the same patient ($P<.05$) (Fig. 5C).

Tissues from premenopausal women expressed more MMP-1 and TIMP-1 compared with postmenopausal women, although these levels did not reach statistical significance (data not shown). Expression of MMP-1 and TIMP-1 among these tissues did not differ by age or sex (data not shown).

DISCUSSION

We found that serosal tissue of several intraperitoneal organs and adhesions expresses MMP-1 and TIMP-1 mRNA and protein, and levels of these substances vary consistently among these tissues. Serosal tissue of ovaries, fallopian tubes, uterus, and parietal peritoneum expresses significantly more MMP-1 mRNA than does serosal tissue of omentum and small and large bowels, and variation in TIMP-1 expression is less distinct. However, expression of TIMP-1 mRNA was 10- to 10,000-fold higher in these tissues than was expression of MMP-1 mRNA. In contrast to mRNA expression, MMP-1 and TIMP-1 protein content varied less among these tissues, although TIMP-1 content was 2- to 10-fold higher than MMP-1.

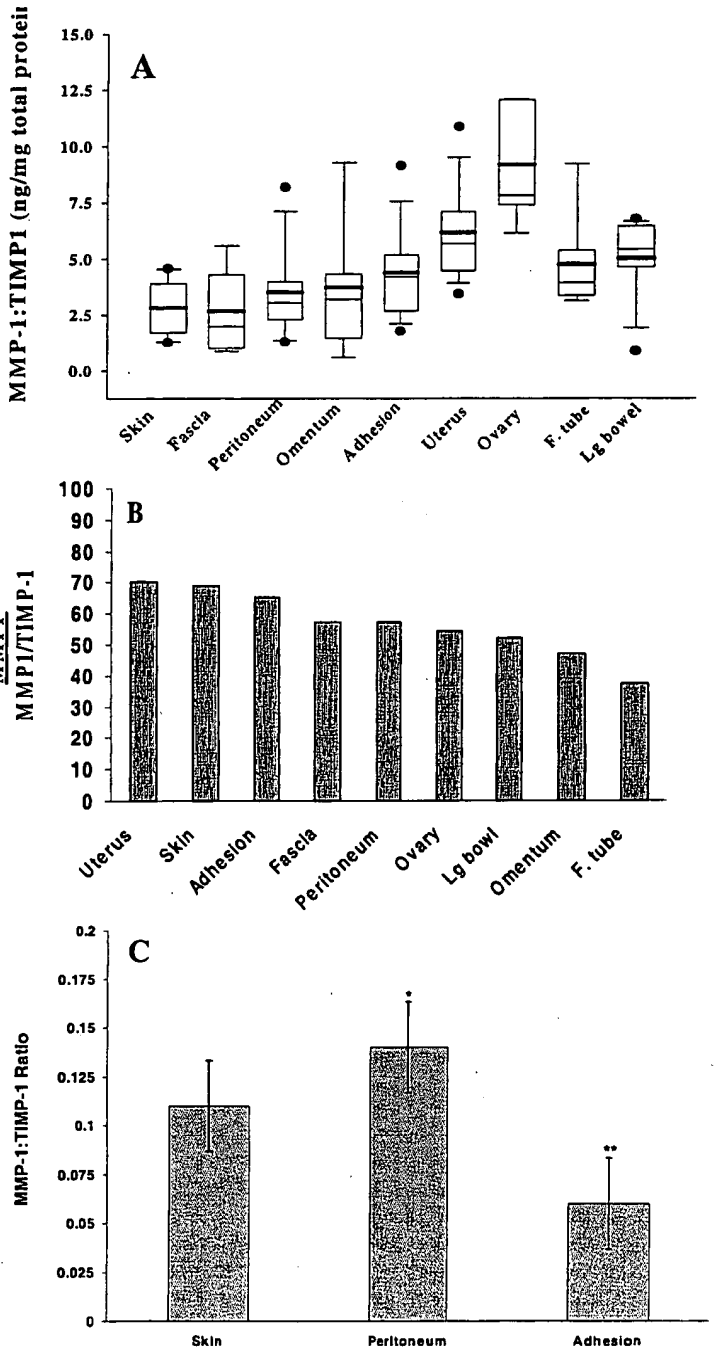
The reasons for the differences in MMP-1 and TIMP-1 expression among these tissues and the disparity between expression of their mRNA and their protein are not clear from our study. However, it is well established that MMP-1 and TIMP-1 are deferentially expressed and regulated at transcriptional and translational levels in other cells and tissues and are influenced by local expression and action of various cytokines, growth factors, and hormones under normal and pathologic conditions (4–12). Considering that these tissues were collected from different organs in a heterogeneous sample, the differences in MMP-1 and TIMP-1 and expression of their mRNA and protein could be due to a combination of the above factors and their microenvironments.

Matrix metalloproteinases are produced as inactive proenzymes that require activation for their local action. In their active form, they become inhibited in part by their physiologic inhibitors, TIMPs (4, 5, 32). Inadequate tissue samples prevented us from analyzing this key property of MMP-1, but our results indicate that the serosal tissue of reproductive organs express more MMP-1 mRNA than do other intraperitoneal organs (e.g., small and large bowels). In addition, the serosal tissue of intraperitoneal organs expresses more TIMP-1 than MMP-1, and adhesions express more TIMP-1 and have a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum.

Although it is well established that peritoneal adhesions form as a result of excessive cellular or tissue injury in many patients who undergo pelvic or abdominal surgeries (1–3), it is poorly understood why adhesions develop in a particular tissue or patient. It is widely accepted that tissue injury alters local expression of various molecules, including MMPs and TIMPs (4, 5). Recent epidemiologic studies indicate that the rate of hospital readmission owing to peritoneal adhesions is

FIGURE 5

(A), Ratio of matrix metalloproteinase (MMP-1) to tissue inhibitor of MMP-1 (*TIMP-1*) in serosal tissue of the parietal peritoneum, uterus, ovaries, fallopian tubes (*F. tube*), large bowels, skin, fascia, omentum, and adhesions. The ratio of MMP-1 to TIMP-1 in skin, fascia, and peritoneum is significantly lower than in other tissues ($P<.001$) and is lower in adhesions than in uterus and ovary ($P=.03$ and .001, respectively). (**B**), Percentiles of MMP-1 associated with TIMP-1 in the tissue extracts, calculated by using the equation indicated on the Y axis. (**C**), Ratio of MMP-1 to TIMP-1 in adhesions, parietal peritoneum, and skin from 10 patients with adhesions. The ratio of these substances in adhesions is significantly lower than that in peritoneum and skin ($P<.05$ for * compared with **).



Chegini. MMP-1 and TIMP-1 in serosal tissue. Fertil Steril 2001.

greater among women who have had gynecologic surgical procedures compared with other pelvic or abdominal surgery (2, 3), and we found that MMP-1 and TIMP-1 expression in serosal tissue of intraperitoneal organs, in particular the reproductive tissues, varied consistently; these findings together suggest that differences in local expression of these molecules may predispose one organ to form more adhesions than others.

Our previous studies on expression of MMP-3, TIMP-2, transforming growth factor βs, and integrins in serosal tissue of intraperitoneal organs provide further support for this idea (24, 33, 34). Of note, the ovarian serosal tissue that expresses different levels of MMP-1 and TIMP-1 undergoes repeated tissue injury during ovulation but heals without scarring, supporting the observations that the type and extent of tissue injury are critical factors in formation of peritoneal adhesions.

We also found that adhesions express more TIMP-1 and have a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum in the same patient, reflecting the ability of adhesions to undergo prolonged tissue remodeling since their formation. A similar observation was made for TIMP-1 content in peritoneal fluid but not in serum of patients with and without adhesions (35), suggesting that local rather than systemic changes predispose patients to development of adhesions. Although other MMPs and TIMPs are also critical to wound healing, MMP-1 (interstitial collagenase), which is produced by epithelial cells, fibroblasts, macrophages, and other cell types in granulation tissue, is present in the wound environment and plays a major role in various stages of healing (4, 5).

Similar to our findings with MMP-3, TIMP-2, transforming growth factor β, and integrin, we found that expression of MMP-1 and TIMP-1 in peritoneal serosal tissue does not differ by age or sex (24, 33, 34). These results further suggest that the molecular environment of serosal tissue of intraperitoneal organs differs from skin, where expression of MMP and TIMP during wound healing has been reported to be age dependent (36, 37). Although further study in a larger sample is required to establish age and sex dependency of expression of these and other molecules in intact peritoneal tissues and during wound healing and adhesion formation, continuous exposure of peritoneal serosal tissue to peritoneal fluid that contains many growth factors and cytokines may account for the differences between intraperitoneal organs and skin.

Matrix metalloproteinases and TIMPs are key regulators of cellular migration, inflammation, angiogenesis, and, most noticeably, the extra-cellular matrix (ECM) turnover (4–22). Unregulated production of these substances at the site of peritoneal injury may alter normal wound healing, leading to formation of adhesions. Several growth factors, cytokines, and hormones that are locally expressed by various wound cells and are present in the peritoneal fluid may regulate expression of MMPs and TIMPs in serosal tissue of the peritoneal organs (4–7, 13, 33–46). For instance, excess production of transforming growth factor β, which causes tissue fibrosis (including peritoneal adhesions), is reported to differentially regulate the expression of MMPs and TIMPs and prevents plasmin generation by increasing the expression of plasminogen activator inhibitor, thereby allowing unopposed deposition of ECM (5, 6, 32, 47–48). Increased local expression of plasminogen activator inhibitor in peritoneal tissue after injury is reported to correspond to higher risk for adhesions (47–49).

In peritoneal mesothelial cells and adhesion fibroblasts, transforming growth factor β differentially regulates expression of α_1-procollagen, fibronectin, TIMP-1, MMP-1, and plasminogen activator inhibitor. Plasmin generation in turn can activate pro-MMPs and latent transforming growth factor β_1 (4, 32), implying that alteration in the expression of any of these molecules after tissue injury can modify their coordinated interactions that lead to adhesion formation. In addition, ovarian steroids and gonadotropins regulate the expression of MMPs and TIMPs in uterus and ovary, respectively (7, 29, 50).

In conclusion, our results indicate that serosal tissue of various intraperitoneal organs and adhesions expresses MMP-1 and TIMP-1 mRNA and protein, levels of which varied consistently among these tissues. This variation in MMP-1 and TIMP-1 expression and the knowledge that tissue injury alters their expression suggest that MMP-1 and TIMP-1 play a key role in normal healing of peritoneal wounds and may predispose an organ to develop more adhesions than others.

APPENDIX

Members of the Peritoneal Healing and Adhesion Multi-University Study (PHAMUS) Group

University of Florida, Department of Obstetrics and Gynecology: Nasser Chegini, Ph.D., Yong Zhao, M.D., Kristina D. Kotseos, B.Sc., Alpa Patel, B.Sc., Chunfeng Ma, M.D., Ph.D., Barbara Bennett, M.D., Frederick W. McLean, M.D., and R. Stan Williams, M.D.; Biomaterials Center, Department of Material Sciences and Engineering: Eugene Goldberg, Ph.D., and Lynn Peck, D.V.M.; Wayne State University: Michael P. Diamond, M.D., Richard Leach, M.D., Ghassen Saed, Ph.D., Karen Collins, B.S., David Svinvich, Ph.D., Frank Yealin, M.D., Ph.D., and Yoram Serokin, M.D.; University of Gothenberg: Lena Holmdahl, M.D., Ph.D., Peter Falk, B.Sc., Marie-Louise Ivarsson, M.D., Ph.D., Maria Hedgren, B.Sc., Maria Bergstrom, M.D., and Ingrid Palmgren, B.Sc.; Genzyme Corporation: James Burns, Ph.D., Kevin Skinner, V.M.D., and Cindy Nickerson, M.S.

Acknowledgments: The authors thank the residents and nurses at the University of Florida, Wayne State University, and Gothenburg University for their efforts in collection of the tissues for this study. They also thank Dr. Hossin Yarandi, College of Nursing, University of Florida, for statistical analysis, and Ruth Ann Klockowski and Landis Young, Department of Obstetrics and Gynecology, University of Florida, for editorial assistance.

Referemces

1. Diamond MP. Prevention of adhesions. In: Gershenson DM, DeCherney AH, Curry SL, eds. Operative gynecology. Philadelphia: WB Saunders, 1993:147–58.
2. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. Lancet 1999;353:1476–80.
3. Lower AM, Hawthorn RJ, Ellis H, O'Brien F, Buchan S, Crowe AM. The impact of adhesions on hospital readmissions over ten years after 8849 open gynecological operations: an assessment from the Surgical and Clinical Adhesions Research Study. Br J Gynecol 2000;107:855–62.
4. Clark RAF. Wound repair. Overview and general consideration. In: Clark RAF, ed. The molecular biology of wound repair. 2nd ed. New York: Plenum Press, 1996;3–50.
5. Parks WC. Matrix metalloproteineases in repair. Wound Repair Regen 1999;7:423–32.
6. Murphy G, Gavrilovic J. Proteolysis and cell migration: creating a path? Curr Opin Cell Biol 1999;11:614–21.
7. Salamonsen L. Matrix metalloproteinases and their tissue inhibitors in endometrial remodeling and menstruation. Reprod Med 1996;53:185–203.
8. Arumugam S, Jang YC, Chen-Jensen C, Gibran NS, Isik FF. Temporal activity of plasminogen activators and matrix metalloproteinases during cutaneous wound repair. Surgery 1999;125:587–93.
9. Madlener M, Parks WC, Werner S. Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. Exp Cell Res 1998;242:201–10.
10. Madlener M. Differential expression of matrix metalloproteinases and their physiological inhibitors in acute murine skin wounds. Arch Dermatol Res 1998;290[Suppl]:S24–9.
11. Kahari VM, Saarialho-Kere U. Matrix metalloproteinases in skin. Exp Dermatol 1997;6:199–213.
12. Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr 1997;7:159–78.
13. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111–22.
14. Blavier L, Henriet P, Imren S, Declerck YA. Tissue inhibitors of matrix metalloproteinases in cancer. Ann N Y Acad Sci 1999;878:108–19.
15. Kostoulas G, Lang A, Nagase H, Baici A. Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases. FEBS Lett 1999;455:286–90.
16. Sang QX. Complex role of matrix metalloproteinases in angiogenesis. Cell Res 1998;8:171–7.
17. Nwomeh BC, Liang HX, Cohen IK, Yager DR. MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. J Surg Res 1999;81:189–95.
18. Saarialho-Kere UK. Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. Arch Dermatol Res 1998;290[Suppl]:S47–54.
19. Savage FJ, Lacombe DL, Boulos PB, Hembry RM. Role of matrix metalloproteinases in healing of colonic anastomosis. Dis Colon Rectum 1997;40:962–70.
20. Tarlton JF, Vickery CJ, Leaper DJ, Bailey AJ. Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. Br J Dermatol 1997;137:506–16.
21. Vaalamo M, Weckroth M, Puolakkainen P, Kere J, Saarinen P, Lauharanta J, et al. Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. Br J Dermatol 1996;135:52–9.
22. Vaalamo M, Leivo T, Saarialho-Kere U. Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, 3, and -4) in normal and aberrant wound healing. Hum Pathol 1999;30:795–802.
23. Sharpe-Timms KL, Zimmer RL, Jolliff WJ, Wright JA, Nothnick WB, Curry TE. Gonadotropin- releasing hormone agonist (GnRH-a) therapy alters activity of plasminogen activators, matrix metalloproteinases, and their inhibitors in rat models for adhesion formation and endometriosis: potential GnRH-a-regulated mechanisms reducing adhesion formation. Fertil Steril 1998;69:916–23.
24. Chegini N, Zhao Y, Kotseos K, Ma C, Bennett B, Diamond MP, Holmdahl L, Skinner K. The expression of matrix metalloproteinase (MMP-3) and tissue inhibitor of MMP (TIMP-2) in serosal tissue of intraperitoneal organs and adhesions. Br J Gynecol 2001.
25. Fukudome K, Fujimoto S, Sato Y, Hisanaga S, Eto T. Peritonitis increases MMP-9 activity in peritoneal effluent from CAPD patients. Nephron 2001;87:35–41.
26. Koks CA, Groothuis PG, Slaats P, Dunselman GA, de Goeij AF, Evers JL. Matrix metalloproteinases and their tissue inhibitors in antegradely shed menstruum and peritoneal fluid. Fertil Steril 2000;73:604–12.
27. Martin J, Yung S, Robson RL, Steadman R, Davies M. Production and regulation of matrix metalloproteinases and their inhibitors by human peritoneal mesothelial cells. Perit Dial Int 2000;20:524–33.
28. Ma C, Tarnuzzer RW, Chegini N. Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in mesothelial cells and their regulation by transforming growth factor β1. Wound Repair Regen 1999;7:477–85.
29. Dou Q, Tarnuzzer RW, Williams RS, Schultz G, Chegini N. Differential expression of matrix metalloproteinases and their tissue inhibitors in leiomyomata: a mechanism for gonadotropin releasing hormone agonist induced tumour regression. Mol Hum Reprod 1997;3:1005–14.
30. Dou Q, Williams RS, Chegini N. Expression of integrin messenger ribonucleic acid in human endometrium: a quantitative reverse transcription polymerase chain reaction study. Fertil Steril 1999;71:347–53.
31. Diamond MP. Reduction of adhesions after uterine myomectomy by Seprafilm membrane (HAL-F): a blinded, prospective, randomized, multicenter clinical study. Seprafilm Adhesion Study Group. Fertil Steril 1996;66:904–10.
32. Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, et al. Mechanisms for pro matrix metalloproteinase activation. APMIS 1999;107:38–44.
33. Chegini N, Kotseos K, ZhaoY, Bennett B, McLean FW, Diamond MP, et al. Differential expression of transforming growth factor β1 and transforming growth factor β3 in serosal tissues of human intraperitoneal organs and peritoneal adhesions. Hum Reprod 2001;16:1291–300.
34. Chegini N, Kotseos K, Ma C, Williams RS, Diamond MP, Holmdahl L, et al. Differential expression of integrin αv and β3 in serosal tissue of human intraperitoneal organs and adhesion. Fertil Steril 2001;75:791–6.
35. Chegini N, Kotseos K, Bennett B, Diamond MP, Holmdahl L, Burns J. Matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in peritoneal fluids and sera and correlation with peritoneal adhesions. Fertil Steril 2001;76:1209–13.
36. Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer RW, Schultz GS, Ferguson MW. Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. Cell Tissue Res 1997;290:581–91.
37. Ashcroft GS, Herrick SE, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW. Human aging impairs injury-induced in vivo expression of tissue inhibitor of matrix metalloproteinases (TIMP)-1 and -2 proteins and mRNA. J Pathol 1997;183:169–76.
38. Chegini N, Rong H, Bennett B, Stone IK. Peritoneal fluid cytokine and eicosanoid levels and their relation to the incidence of peritoneal adhesion. J Soc Gynecol Invest 1999;6:153–7.
39. Mulayim N, Arici A. The relevance of the peritoneal fluid in endometriosis-associated infertility. Hum Reprod 1999;14[Suppl 2]:67–76.
40. Simeon A, Monier F, Emonard H, Wegrowski Y, Bellon G, Monboisse JC, et al. Fibroblast-cytokine-extracellular matrix interactions in wound repair. Curr Top Pathol 1999;93:95–101.
41. Chegini N. The role of growth factors in peritoneal healing: transforming growth factor β (TGF-β). Eur J Surg 1997;163[Suppl 577]:17–23.
42. Holmdahl L, Kotseos K, Bergstrom M, Iversson ML, Falk P, Chegini N. Overproduction of TGF-β is associated with adhesion formation and fibrinolytic impairment. Surgery 2001;129:626–32.
43. Ghellai AM, Stucchi AF, Chegini N, Ma C, Andry CD, Kaseta JM, et al. Role of transforming growth factor beta-1 in peritonitis-induced adhestions. Gastrointest Surg 2000;4:316–23.
44. Ma C, Chegini N. Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-β1. Mol Hum Reprod 1999;5:950–4.
45. Saed GM, Zhang W, Chegini N, Holmdahl L, Diamond MP. Alteration of type I and III collagen expression in human peritoneal mesothelial cells in response to hypoxia and transforming growth factor-β_1. Wound Repair Regen 1999;7:504–10.
46. Flak P, Ma C, Chegini N, Holmdahl L. Differential regulation of mesothelial cell fibrinolysis by transforming growth factor beta 1 (TGF-β_1). Scan J Clin Lab Invest 2000;60:439–48.
47. Thompson J. Pathogenesis and prevention of adhesion formation. Dig Surg 1998;15:153–7.
48. Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg 1997;163:24–31.
49. Edelstam G, Lecander I, Larsson B, Astedt B. Fibrinolysis in the peritoneal fluid during adhesions, endometriosis and ongoing pelvic inflammatory disease. Inflammation 1998;22:341–51.
50. Hulboy DL, Rudolph LA, Matrisian LM. Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod 1997;3:27–45.

Exhibit N

FERTILITY AND STERILITY®
VOL. 76, NO. 6, DECEMBER 2001

Published by Elsevier Science Inc.
Printed on acid-free paper in U.S.A.

Matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in peritoneal fluids and sera and correlation with peritoneal adhesions

Nasser Chegini, Ph.D.,[a] Kristina Kotseos, B.Sc.,[a] Barbara Bennett, M.D.,[a] Michael P. Diamond, M.D.,[b] Lena Holmdahl, M.D., Ph.D.,[c] James Burns, Ph.D.,[d] and the Peritoneal Healing and Adhesion Multiuniversity Study (PHAMUS) Group

Institute for Wound Research, University of Florida, Gainesville, Florida

Objective: To assess the presence of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP (TIMP-1) in peritoneal fluid and serum of subjects with and without adhesions.

Design: Cross-sectional study.

Setting: Academic research centers.

Patient(s): Sixty-three patients who underwent abdominal/pelvic surgery.

Intervention(s): MMP-1, TIMP-1, and MMP-1–TIMP-1 complex content.

Main Outcome Measure(s): ELISA.

Result(s): Peritoneal fluids (PF) and sera of subjects with and without peritoneal adhesions contain MMP-1, TIMP-1, and MMP-1–TIMP-1 complex at varying levels with 10- to 100-fold higher TIMP-1 than MMP-1. Compared with serum, PF contains a lower level of MMP-1 in subjects with mild adhesions and without adhesions, higher TIMP-1 in subjects with extensive adhesions, and lower MMP-1–TIMP-1 complex in subjects with moderate adhesions. However, the serum and PF content of MMP-1, TIMP-1, and MMP-1–TIMP-1 complex was not statistically different among subjects with or without adhesions, with the exception of TIMP-1 in PF of subjects with extensive adhesions. MMP1–TIMP-1 ratio indicates that a major portion of MMP-1 is in complex with TIMP-1. There was no age- or gender-dependent difference in MMP-1 and TIMP-1 content in serum or PF.

Conclusion(s): Despite differences in MMP-1 and TIMP-1 levels in serum and PF of subjects with extensive and moderate adhesions, there is no correlation between MMP-1 and TIMP-1, with the exception of higher TIMP-1 in PF of subjects with extensive adhesions. (Fertil Steril® 2001;76:1207–11. ©2001 by American Society for Reproductive Medicine.)

Key Words: MMP-1, TIMP-1 serum, peritoneal fluid, peritoneal adhesion

Received December 7, 2000; revised and accepted June 11, 2001.

Reprint requests: Nasser Chegini, Ph.D., University of Florida, Department of Obstetrics and Gynecology, Box 100294, Gainesville, Florida 32610-0294 (FAX: 352-392-6994; E-mail: cheginin@obgyn.ufl.edu).

[a] Department of Obstetrics and Gynecology, University of Florida.

[b] Department of Obstetrics and Gynecology, Wayne State University, Detroit, Michigan.

[c] Department of Surgery, Gothenberg University, Gothenberg, Sweden.

[d] Genzyme Corporation, Cambridge, Massachusetts.

0015-0282/01/$20.00
PII S0015-0282(01)02874-6

Proteolysis of extracellular matrix (ECM) is critical to inflammation, cellular migration, angiogenesis, proliferation, and tissue remodeling, processes that are integral parts of normal wound healing (1–3). Several classes of endoproteinases such as matrix metalloproteinases (MMPs) with a broad range of proteolytic activity have been identified, and patterns of their expression indicate a low level of expression in normal adult tissue, with the exception of tissues that normally undergo extensive remodeling, such as endometrium during the menstrual cycle and tissues during the early phase of wound healing (1–8).

Furthermore, elevated expression of MMPs has been associated with various connective tissue disorders; inflammation; and impaired, nonhealing wounds (1, 5–14). The proteolytic activity of MMPs is controlled in part by naturally occurring inhibitors, the tissue inhibitor of MMPs (TIMPs). They consist of TIMP-1 to TIMP-4 and are expressed in a wide variety of tissues, and in addition to inhibiting MMP proteolytic activity, they regulate cell migration, angiogenesis, and cell growth (1, 2, 11, 15–17).

Recent reports also indicate that MMPs and TIMPs are expressed by peritoneal mesothelial cells, adhesion fibroblasts, and serosal tissue of various peritoneal organs and are detectable in

peritoneal fluid of surgically induced adhesions in rats and humans with endometriosis and peritonitis (18–25). These results suggest that MMPs and TIMPs may influence the outcome of peritoneal wound healing and the incidence of adhesion formation after cellular or tissue injury induced by surgical procedure, infection, and inflammation. The objective of this study was to determine whether alteration in peritoneal MMP-1 and TIMP-1 correlates with the presence of peritoneal adhesions and whether the changes are reflected systemically in serum of these subjects.

MATERIALS AND METHODS

Peritoneal fluids (PF) and serum were collected from 63 subjects with and without adhesions who were undergoing abdominal or pelvic surgeries. Of these patients 51 were female and 12 were male, ranging in age from 24 to 83 years. Of the female patients, 42 were premenopausal, of whom 23 had undergone previous surgical procedures including cesarean sections, bilateral tubal intervention, appendectomy, ovarian cystectomy, hysterectomy, and/or treatment for endometriosis, and 9 were postmenopausal. Male patients were scheduled to undergo various gastrointestinal surgical procedures.

On the basis of the premenopausal patient's last menstrual period and endometrial histology, 19 were in the proliferative phase and 23 were in the secretory phase of the menstrual cycle. Pelvic findings at surgery were used to assess the type of adhesions, which were classified based on their severity as previously described (26). All patients gave informed written consent before undergoing surgery. The institutional review board approvals were obtained from the University of Florida, Wayne State University, and Gothenburg University before initiation of the study.

Peritoneal fluids were not included in the assay if grossly contaminated; therefore, PF from 26 subjects were included in this study. Aliquots of peritoneal fluids and sera were stored at −80°C until assayed. Total protein content was determined using a standard protein assay (Bio-Rad, Hercules, CA). An equal amount of total protein from each preparation was subjected to ELISA measuring total MMP-1 (free and complex with TIMP-1 but not with α2-macroglobulin), total TIMP-1 (free and complex with MMPs), and MMP-1–TIMP-1 complex (activated MMP-1 in complex with TIMP-1), respectively. Human-specific MMP-1, TIMP-1, and MMP-1–TIMP-1 complex ELISA kits with limits of detection of 1.7, 1.3, and 1.5 ng/mL, respectively, were purchased from Oncogene Sciences, Inc. (Cambridge, MA). All measurements were performed in duplicate, and the results are reported as mean ± SEM per milligram of total protein.

For statistical analysis, unpaired Student's t test and Kruskal-Wallis one-way analysis of variance with Dunn test using computer software program SigmaStat (Jandel Co, San Rafael, CA) was performed. A probability level of $P<.05$ was considered significant.

RESULTS

All the PF and sera assayed contained MMP-1, TIMP-1, and MMP-1–TIMP-1 complex at varying levels with 10- to 100-fold higher TIMP-1 than MMP-1. Comparatively, PF contains less MMP-1 ($P=.01$) and MMP-1–TIMP-1 complex ($P=.03$) and more TIMP-1 ($P=.02$) than sera, with substantial variations among the subjects (Fig. 1). The serum levels of MMP-1 were higher in subjects with moderate adhesions and without adhesions compared with PFs ($P<.05$); however, their levels in serum or PF among subjects with adhesions were not significantly different compared with those in subjects without adhesions (Fig. 2A).

The level of TIMP-1 in PF and sera of subjects with different rates of adhesions and without adhesions is shown in Fig. 2B. These levels were not significantly different between and among the groups, with the exception of higher TIMP-1 in PF of subjects with extensive adhesions compared with those without adhesions ($P<.05$). The levels of MMP-1–TIMP-1 complex were low and with the exception of higher levels in sera of subjects with moderate adhesions ($P<.05$), these levels were not significantly different between sera and PF among these groups with or without adhesions. In addition, the MMP-1–TIMP-1 ratio indicates that a major portion of MMP-1 is in complex with TIMP-1 (data not shown).

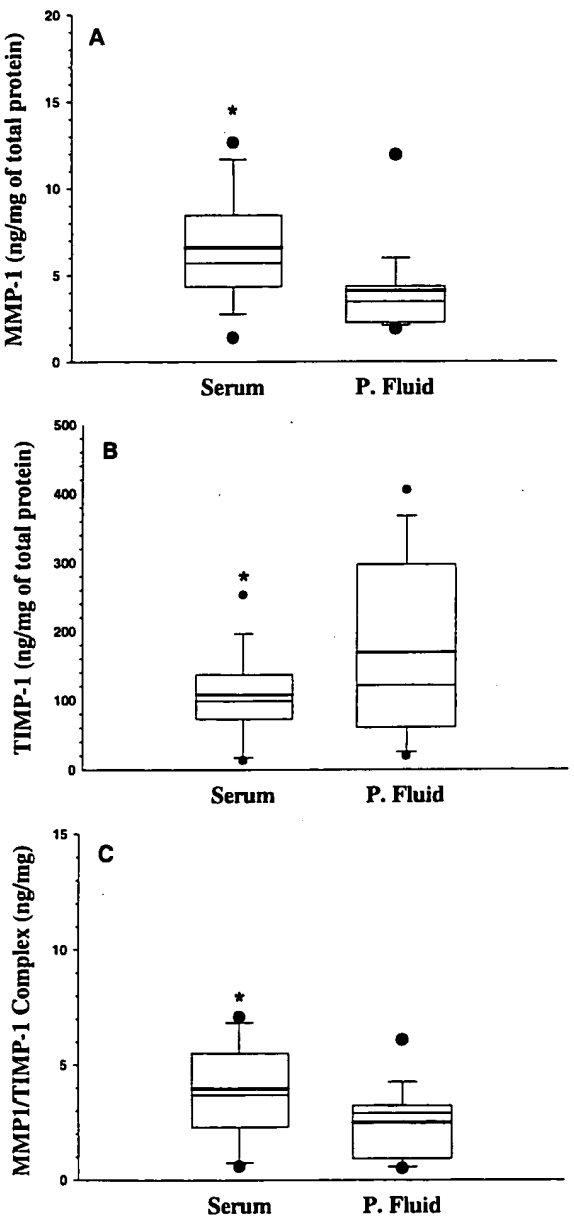
DISCUSSION

In the present study, we have provided evidence that the peritoneal fluid levels of TIMP-1 in subjects with extensive adhesions, but not those with moderate or mild adhesions, are higher compared with those in subjects without adhesions. Such differences were not observed for MMP-1 in PF or for MMP-1 and TIMP-1 in sera, implicating local rather than systemic alterations in TIMP-1 expression in subjects with adhesion. Interestingly, there was an inverse relation between TIMP-1 and MMP-1 levels in serum and PF of subjects with extensive and moderate adhesions, but not in other groups, with or without adhesions. In addition TIMP-1 levels exceeded that of MMP-1 by 10- to 100-fold, suggesting that TIMP-1 may play a more critical role in adhesion development if their levels are altered after peritoneal tissue injury.

The most likely source of MMP-1 and TIMP-1 in PF is the peritoneal mesothelial cells that express MMP-1 and TIMP-1 mRNA and protein and other MMPs and TIMPs (18, 19). However, we have shown a substantial variation in MMP-1 and TIMP-1 expression among the serosal tissue of intraperitoneal organs and adhesions, with TIMP-1 expres-

FIGURE 1

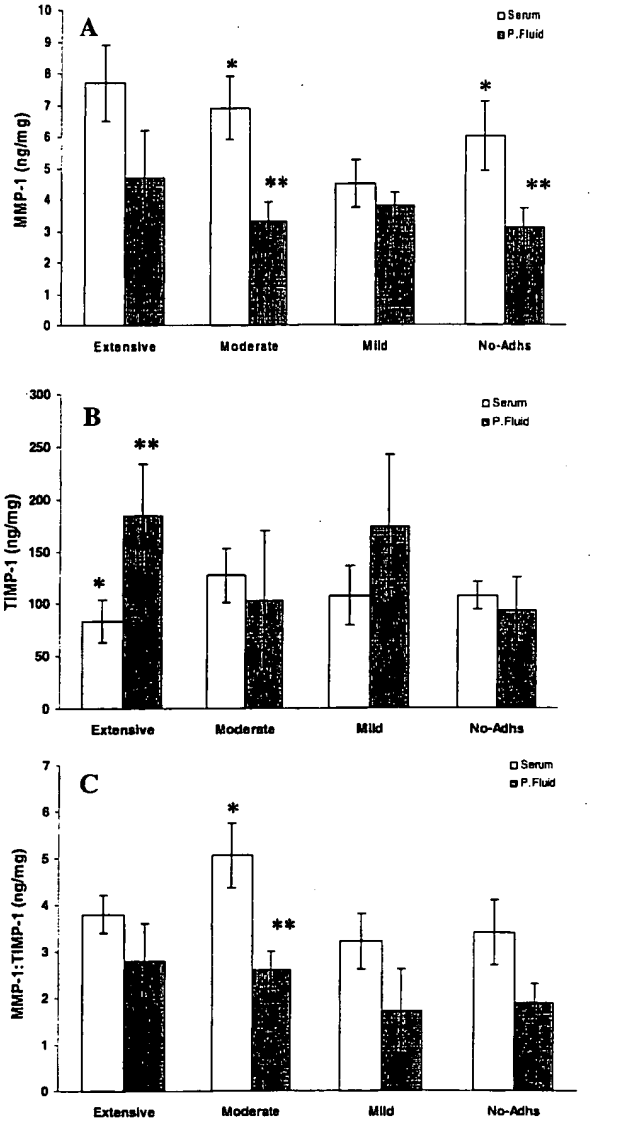
Notched box plots showing the distribution of MMP-1 (**A**), TIMP-1 (**B**), and MMP1/TIMP1 complex (**C**) in serum and peritoneal fluid. The vertical line within the box boundaries represents the distribution of the middle 50%, the thin horizontal lines within the boxes are the median, and the thick lines are the arithmetic mean. The notches show the 95th percentile and the error bars, the 90th and 10th percentiles. In **A, B,** and **C,** serum level (*) differs from peritoneal fluid level (P. Fluid; $P=.01$, .02, and .03, respectively).



Chegini. MMP/TIMP and adhesions. Fertil Steril 2001.

FIGURE 2

The levels (mean ± SEM) of MMP-1 (**A**), TIMP-1 (**B**), and MMP-1/TIMP1 complex (**C**) in serum and PF of subjects with extensive, moderate, and mild adhesions, and without adhesions. There was no significant difference in MMP-1, TIMP-1, or MMP-1–TIMP-1 complex levels among the groups, with the exception of TIMP-1 in PF of subjects with extensive adhesions. *, **Statistically significant difference between these groups ($P<.05$). No-Adhs = no adhesion.



Chegini. MMP/TIMP and adhesions. Fertil Steril 2001.

sion exceeding that of MMP-1 by ~100- to 10,000-fold and by 2- to 10-fold at mRNA and protein levels, respectively (20).

A higher TIMP-1 content and low MMP-1–TIMP-1 ratio in PF imply that the peritoneal environment favors matrix deposition rather than degradation at least in subjects in our study. Similar to the serosal tissues, most of the MMP-1 detected in the PF and serum was in complex with TIMP-1. Whether such an environment is maintained immediately

after peritoneal injury and during the early phase of peritoneal healing has not yet been determined. However, in surgically induced peritoneal injury in rats, the PF levels of MMP and MMP-1 activity before injury and 7 weeks after injury has been reported to be similar, although adhesions scores showed positive correlation with MMP activity (22).

In contrast, the TIMP-1 content in PF and sera of women with endometriosis, a condition that is associated with peritoneal inflammation, has been reported to be significantly lower compared with that in disease-free women (23). Furthermore, in subjects with peritonitis, the peritoneal effluent levels of latent and activated MMP-9 and TIMP-1, but not MMP-2 and TIMP-2, have been reported to be higher at the onset of peritonitis, a condition that causes peritoneal adhesion, than during the recovery phase of peritonitis and/or in normal controls (24).

A lack of correlation between MMP-1 activity in PF and peritoneal injury in rats (23) is most likely to be caused by timing of the second look at 7 weeks, because elevated expression of various factors at the site of peritoneal wounds and in PF has been reported to peak during the 1st week postinjury and to return to normal levels thereafter (27–29). Such pattern of expression has also been reported for MMPs and TIMPs in dermal wound healing whose expression and activity increases during the 1st week after injury, with the exception of chronic and impaired wounds (1, 5, 6, 10–17).

Because peritoneal adhesions form as a result of excessive cellular migration and matrix deposition, and MMPs and TIMPs are considered as key regulators of these events, alterations of their expression could play a critical role in adhesion development. In particular, MMPs and TIMPs modulate cellular migration, inflammation, angiogenesis, cell growth, and most noticeably the extracellular matrix turnover, events that are critical in the outcome of normal wound healing and disorders such as delayed and chronic wounds and scar formation (1–17). Further studies of the role of MMPs and TIMPs in these events involving peritoneal mesothelial and adhesion fibroblasts are needed to better our understanding of the peritoneal environment that leads to adhesion development.

Interestingly, we found a lack of correlation between PF and serum levels of MMP-1 and TIMP-1 and that of age and gender and between premenopausal and postmenopausal women, a condition that was observed for the expression of MMP, TIMP, TGF-β, and integrin in serosal tissue of several intraperitoneal organs (20, 21, 30, 31). These results support the clinical observations that a vast number of subjects who undergo surgical procedures develop adhesions, although certain populations, regardless of age and gender, develop more adhesions than others (32, 33).

These findings suggest that factors present in peritoneal fluid may directly or indirectly influence the peritoneal environment, which is distinct from other wounds, including dermal wounds in which age-dependent healing has been observed (1, 2). This includes the influence of a variety of cytokines, growth factors, and hormones that are expressed by the serosal tissue of these organs and are present in peritoneal fluids and that also regulate the expression of MMPs and TIMPs (1, 2, 4, 13, 27–29, 34).

In summary, these results indicate that peritoneal fluid and serum of subjects with and without adhesions contain MMP-1 and TIMP-1, with higher TIMP-1 levels in PF of subjects with adhesions. The results suggest that local rather than systemic alteration involving TIMP-1 may provide a favorable condition that predisposes an individual to develop adhesions and further supports their establishment after peritoneal injury.

Acknowledgments: The authors would like to acknowledge the contributions of many residents and nurses at the Universities of Florida, Wayne State, and Gothenberg for their efforts in collection of samples for this study; they also thank RuthAnn Klockowski, University of Florida, Department of Obstetrics and Gynecology, for editorial assistance.

The Peritoneal Healing and Adhesion Multi University Study (PHAMUS) Group includes the following investigators: Nasser Chegini, Ph.D., Yong Zhao, M.D., Kristina D. Kotseos, B.Sc, Alpa Patel, B.Sc, Chunfeng Ma, M.D., Ph.D., Barbara Bennett, M.D., Frederick W. McLean, M.D., and R. Stan Williams, M.D., from the Department of Obstetrics and Gynecology, University of Florida; Eugene Goldberg, Ph.D., and Lynn Peck, D.V.M., from the Biomaterials Center, Department of Material Sciences and Engineering, University of Florida; Michael P. Diamond, M.D., Richard Leach, M.D., Ghassen Saed, Ph.D., Karen Collins, B.S., David Svinvich, Ph.D., Frank Yealin, M.D., Ph.D., and Yoram Serokin, M.D., from Wayne State University; Lena Holmdahl, M.D., Ph.D., Peter Falk, M.S., Marie-Louise Ivarsson, M.D, Ph.D., Maria Hedgren, B.Sc., Maria Bergstrom, M.D., and Ingrid Palmgren, B.Sc. from University of Gothenberg, Gothenberg, Sweden; and James Burns, Ph.D., Kevin Skinner, V.M.D., and Cindy Nickerson, M.S, from Genzyme Co.

References

1. Parks WC. Matrix metalloproteinases in repair. Wound Rep Regen 1999;7:423–32.
2. Clark RAF. Wound repair. Overview and general consideration. In: Clark RAF, ed. The molecular biology of wound repair. 2nd ed. New York: Plenum Press, 1996:3–50.
3. Murphy G, Gavrilovic J. Proteolysis and cell migration: creating a path? Curr Opin Cell Biol 1999;11:614–21.
4. Salamonsen L. Matrix metalloproteinases and their tissue inhibitors in endometrial remodeling and menstruation. Reprod Med 1996;53:185–203.
5. Arumugam S, Jang YC, Chen-Jensen C, Gibran NS, Isik FF. Temporal activity of plasminogen activators and matrix metalloproteinases during cutaneous wound repair. Surgery 1999;125:587–93.
6. Madlener M, Parks WC, Werner S. Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. Exp Cell Res 1998;242:201–10.
7. Kahari VM, Saarialho-Kere U. Matrix metalloproteinases in skin. Exp Dermatol 1997;6:199–213.
8. Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr 1997;7:159–78.
9. Sang QX. Complex role of matrix metalloproteinases in angiogenesis. Cell Res 1998;8:171–7.
10. Nwomeh BC, Liang HX, Cohen IK, Yager DR. MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. J Surg Res 1999;81:189–95.

11. Saarialho-Kere UK. Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. Arch Dermatol Res 1998;290[Suppl]:S47–54.
12. Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H, Larjava H. Expression of matrix metalloproteinase-2 and -9 during early human wound healing. Lab Invest 1994;70:176–82.
13. Savage FJ, Lacombe DL, Boulos PB, Hembry RM. Role of matrix metalloproteinases in healing of colonic anastomosis. Dis Colon Rectum 1997;40:962–70.
14. Tarlton JF, Vickery CJ, Leaper DJ, Bailey AJ. Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. Br J Dermatol 1997;137:506–16.
15. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111–22.
16. Kostoulas G, Lang A, Nagase H, Baici A. Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases. FEBS Lett 1999;455:286–90.
17. Vaalamo M, Leivo T, Saarialho-Kere U. Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) in normal and aberrant wound healing. Hum Pathol 1999;30:795–802.
18. Ma C, Tamuzzer RW, Chegini N. Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in mesothelial cells and their regulation by transforming growth factor β1. Wound Rep Regen 1999;7:477–85.
19. Martin J, Yung S, Robson RL, Steadman R, Davies M. Production and regulation of matrix metalloproteinases and their inhibitors by human peritoneal mesothelial cells. Perit Dial Int 2000;20:524–33.
20. Chegini N, Kotseos K, Zhao Y, Ma C, McLean F, Diamond MP, et al. Expression of matrix metalloproteinase and tissue inhibitor of MMP in serosal tissue of intraperitoneal organs and adhesions. Fertil Steril 2001;76:1214–21.
21. Chegini N, Zhao Y, Kotseos, K. Bennett B, Diamond MP, Holmdahl L, et al. Matrix metalloproteinase (MMP-3), tissue inhibitor of MMP (TIMP-2) expression in intraperitoneal environment and their relation to adhesion development. Br J Gynecol 2001. Submitted.
22. Sharpe-Timms KL, Zimmer RL, Jolliff WJ, Wright JA, Nothnick WB, Curry TE. Gonadotropin-releasing hormone agonist (GnRH-a) therapy alters activity of plasminogen activators, matrix metalloproteinases, and their inhibitors in rat models for adhesion formation and endometriosis: potential GnRH-a-regulated mechanisms reducing adhesion formation. Fertil Steril 1998;69:916–23.
23. Sharpe-Timms KL, Keisler LW, McIntush EW, Keisler DH. Tissue inhibitor of metalloproteinase-1 concentrations are attenuated in peritoneal fluid and sera of women with endometriosis and restored in sera by gonadotropin-releasing hormone agonist therapy. Fertil Steril 1998; 69:1128–34.
24. Fukudome K, Fujimoto S, Sato Y, Hisanaga S, Eto T. Peritonitis increases MMP-9 activity in peritoneal effluent from CAPD patients. Nephron 2001;87:35–41.
25. Koks CA, Groothuis PG, Slaats P, Dunselman GA, de Goeij AF, Evers JL. Matrix metalloproteinases and their tissue inhibitors in antegradely shed menstruum and peritoneal fluid. Fertil Steril 2000;73:604–12.
26. Diamond MP. Reduction of adhesions after uterine myomectomy by Seprafilm membrane (HAL-F): a blinded, prospective, randomized, multicenter clinical study. Seprafilm Adhesion Study Group. Fertil Steril 1996;66:904–10.
27. Chegini N. The role of growth factors in peritoneal healing: transforming growth factor β (TGF-β). Eur J Surg 1997;163(Suppl 577):17–23.
28. Mulayim N, Arici A. The relevance of the peritoneal fluid in endometriosis-associated infertility. Hum Reprod 1999;14(Suppl 2):67–76.
29. Rong H, Tang X-M, Zhao Y, Juneja SC, Fay MF, Williams RS, et al. Peritoneal exposure to surgical glove powders differentially modulate the inflammatory and immune related cytokines production during surgically-induced adhesion formation. Wound Rep Regen 1997;5:89–96.
30. Chegini N, Kotseos K, Zhao Y, Bennett B, McLean FW, Diamond MP, et al. Differential expression of TGF-β1 and TGF-β3 in serosal tissues of human intraperitoneal organs and peritoneal adhesions. Hum Reprod 2001;16:1291–300.
31. Chegini N, Kotseos K, Ma C, Williams RS, Diamond MP, Holmdahl L, et al. Differential expression of integrin αv and β3 in serosal tissue of human intraperitoneal organs and adhesion. Fertil Steril 2001;75: 791–6.
32. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. Lancet 1999;353:1476–80.
33. Lower AM, Hawthorn RJ, Ellis H, O'Brien F, Buchan S, Crowe AM. The impact of adhesions on hospital readmissions over ten years after 8849 open gynaecological operations: an assessment from the Surgical and Clinical Adhesions Research Study. Br J Gynecol 2000;107:855–62.
34. Chegini N, Rong H, Bennett B, Stone IK. Peritoneal fluid cytokine and eicosanoid levels and their relation to the incidence of peritoneal adhesion. J Soc Gynecol Invest 1999;6:153–7.